

A

Practitioner's Docket No. 47728

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): **Eduardo MARBAN**

WARNING: 37 CFR 1.41(a)(1) points out:

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(t) is filed supplying or changing the name or names of the inventor or inventors."

For (title): **SOMATIC TRANSFER OF MODIFIED GENES TO PREDICT DRUG EFFECTS**

CERTIFICATION UNDER 37 C.F.R. 1.10*

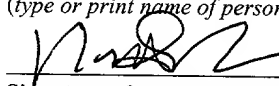
*(Express Mail label number is **mandatory**.)*

(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on this date **November 5, 1998**, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number EL117825129US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Peter F. Corless

(type or print name of person mailing paper)



Signature of person mailing paper

WARNING: Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. 1.10(b).
"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

1. Type of Application

This new application is for a(n)

(check one applicable item below)

☒ Original (nonprovisional)

☐ Design

☐ Plant

WARNING: *Do not use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

WARNING: *Do not use this transmittal for the filing of a provisional application.*

NOTE: *If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.*

☐ Divisional.

☒ Continuation.

☐ Continuation-in-part (C-I-P).

2. Benefit of Prior U.S. Application(s) (35 U.S.C. 119(e), 120, or 121)

NOTE: *A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. 112. Each prior application must also be:*

(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America, or

(ii) Complete as set forth in § 1.51(b); or

(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or

(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).

37 CFR 1.78(a)(1).

NOTE *If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional*

application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

WARNING: If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. 120, 121 or 365(c). (35 U.S.C. 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.

WARNING: When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application **must** be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).

☒ The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed

A. Required for Filing Date under 37 C.F.R. 1.53(b) (Regular) or 37 C.F.R. 1.153 (Design) Application

41 Pages of Specification

4 Pages of Claims

25 Sheets of Drawing

☒ Formal

☐ Informal

B. Other Papers Enclosed

1 Pages of Abstract

 Other

WARNING: **DO NOT** submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. 1.84, see Notice of March 9, 1988 . . . (1990 O.G. 57-62).

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page " 37 C.F.R. 1.84(c)).

(complete the following, if applicable)

- ☐ The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. 1.84(b).

4. Additional Papers Enclosed

- ☐ Preliminary Amendment
☐ Information Disclosure Statement (37 C.F.R. 1.98)
☐ Form PTO-1449 (PTO/SB/08A and 08B)
☐ Citations
☐ Declaration of Biological Deposit
☐ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.
☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative
☐ Special Comments
☐ Other

5. Declaration or Oath

NOTE: *A newly executed declaration is not required in a continuation or divisional application provided the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47 then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 CFR 1.63(d).*

NOTE: *A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name, including the family name, and at least one given name without abbreviation together with any other given name or initial, and the residence, post office address and country of citizenship of each inventor and state whether the inventor is a sole or joint inventor. 37 CFR 1.63(a)(1)-(4).*

- ☐ Enclosed

Executed by

(check **all** applicable boxes)

- ☐ inventor(s).
☐ legal representative of inventor(s). 37 CFR 1.42 or 1.43.
☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.
☐ This is the petition required by 37 CFR 1.47 and the statement required by 37 CFR 1.47 is also attached. See item 13 below for fee.

- ☒ Not Enclosed.

NOTE: *Where the filing is a completion in the U.S. of an International Application, or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a*

continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

☒ Application is made by a person authorized under 37 C.F.R. 1.41(c) on behalf of all the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 CFR 1.16(e), can be filed subsequently).

NOTE: It is important that all the correct inventor(s) are named for filing under 37 CFR 1.41(c) and 1.53(b).

☐ Showing that the filing is authorized.
(not required unless called into question. 37 CFR 1.41(d))

6. Inventorship Statement

WARNING: If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.

The inventorship for all the claims in this application are:

☐ The same.

or

☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,
☐ is submitted.
☐ will be submitted.

7. Language

NOTE: An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of \$130.00 required by 37 CFR 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 CFR 1.52(d).

☒ English

☐ Non-English

☐ The attached translation includes a statement that the translation is accurate. 37 C.F.R. 1.52(d).

8. Assignment

☐ An assignment of the invention to _____

☐ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

☐ was filed in the parent application

☒ will follow.

NOTE: "If an assignment is submitted with a new application, send two separate letters—one for the application and one for the assignment" Notice of May 4, 1990 (1114 O.G. 77-78).

WARNING: A newly executed "STATEMENT UNDER 37 CFR 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.

9. Certified Copy

Certified copy(ies) of application(s)

<u>Country</u>	<u>Appln. No.</u>	<u>Filed</u>
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from which priority is claimed

☐ was filed in parent application No. 08/911,445.

☐ will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 CFR 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

10. Fee Calculation (37 C.F.R. 1.16)

A. ☒ Regular application

CLAIMS AS FILED

Claims	Number Filed	Basic Fee Allowance	Number Extra	Rate	Basic Fee 37 C.F.R. 1.16(a)
Total Claims (37 CFR 1.16(c))	33	- 20 = 13	x	\$ 22.00	\$790.00 \$286.00
Independent Claims (37 CFR 1.16(b))	6	- 3 = 3	x	\$82.00	\$246.00
Multiple Dependent Claim(s), if any (37 CFR 1.16(d))			+	\$270.00	\$270.00

- ☐ Amendment cancelling extra claims is enclosed.
☐ Amendment deleting multiple-dependencies is enclosed.
☐ Fee for extra claims is not being paid at this time.

NOTE: *If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 CFR 1.16(d).*

Filing Fee Calculation \$ 1,592.00

- B.** ☐ Design application
(\$330.00—37 CFR 1.16(f))
Filing Fee Calculation \$ _____
- C.** ☐ Plant application
(\$540.00—37 CFR 1.16(g))
Filing Fee Calculation \$ _____

11. Small Entity Statement(s)

- ☐ Statement(s) that this is a filing by a small entity under 37 CFR 1.9 and 1.27 is (are) attached.

WARNING: *"Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 CFR 1.28(a)(2).*

(complete the following, if applicable)

- ☒ Status as a small entity was claimed in prior application
60/064,893, filed on November 7, 1997 from which benefit is being claimed for this application under:

- 35 U.S.C. § ☐ 119(e),
☐ 120,
☐ 121,
☐ 365(c),

and which status as a small entity is still proper and desired.

☒ A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of **A**, **B** or **C** above) \$ 796.00

NOTE: Any excess of the full fee paid will be refunded if a small entity status is established refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 CFR 1.28(a).

12. Request for International-Type Search (37 C.F.R. 1.104(d))

(complete, if applicable)

☐ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made at This Time

☒ Not Enclosed

☒ No filing fee is to be paid at this time.
(This and the surcharge required by 37 C.F.R. 1.16(e) can be paid subsequently.)

☐ Enclosed

☐ Filing fee \$ _____

☐ Recording assignment
(\$40.00; 37 C.F.R. 1.21(h))
(See attached "COVER SHEET FOR
ASSIGNMENT ACCOMPANYING NEW
APPLICATION.") \$ _____

☐ Petition fee for filing by other than
all the inventors or person on behalf
of the inventor where inventor
refused to sign or cannot be reached
(\$130.00; 37 C.F.R. 1.47 and 1.17(i)) \$ _____

☐ For processing an application with a
specification in a non-English language
(\$130.00; 37 C.F.R. 1.52(d) and 1.17(k)) \$ _____

☐ Processing and retention fee
(\$130.00; 37 C.F.R. 1.53(d) and 1.21(l)) \$ _____

☐ Fee for international-type search report
(\$40.00; 37 C.F.R. 1.21(e)) \$ _____

NOTE: 37 CFR 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 CFR 1.53(f) and this, as well as the changes to 37 CFR 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total Fees Enclosed

\$_____

14. Method of Payment of Fees

- ☐ Check in the amount of \$_____.
- ☐ Charge Account No. _____ in the amount of \$_____.
A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 CFR 1.22(b).

15. Authorization to Charge Additional Fees

WARNING: If no fees are to be paid on filing, the following items should not be completed.

WARNING: Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.

- ☐ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No._____.
- ☐ 37 C.F.R. 1.16(a), (f) or (g) (filing fees)
- ☐ 37 C.F.R. 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 CFR 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

- ☐ 37 C.F.R. 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)
- ☐ 37 CFR 1.17(a)(1)-(5) (extension fees pursuant to § 1.136(a).
- ☐ 37 C.F.R. 1.17 (application processing fees)

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 CFR 1.136(a)(3).

- ☐ 37 C.F.R. 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 CFR 1.311(b))

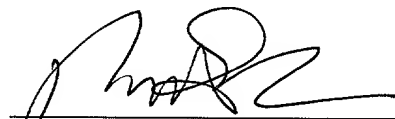
NOTE: 37 CFR 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . issue fee." From the wording of 37 CFR 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

16. Instructions as to Overpayment

NOTE: "... Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 CFR 1.26(a).

☒ Credit Account No. 04-1105

☐ Refund



SIGNATURE OF PRACTITIONER

Reg. No. 33,860

Peter F. Corless

(type or print name of practitioner)

Tel. No.: (617) 523-3400

Dike, Bronstein, Roberts & Cushman, LLP

130 Water Street

P.O. Address

Customer No.:

Boston, MA 02109

☒ **Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

☒ Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

☐ Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added _____

☐ Plus added pages deleting names of inventor(s) named on prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added _____

☐ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added _____

☐ **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

☐ This transmittal ends with this page.

ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

NOTE: See 37 CFR 1.78.

17. Relate Back

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. 120, 121 or 365(c). (35 U.S.C. 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

☒ Amend the specification by inserting, before the first line, the following sentence:

A. 35 U.S.C. 119(e)

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

☒ "This application claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:

FILING DATE

60/064,893

November 7, 1997

"

"

"

B. 35 U.S.C. 120, 121 and 365(c)

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).

☐ "This application is a

☐ continuation

☐ continuation-in-part

☐ divisional

of copending application(s)

☐ application number _____”

☐ International Application _____ filed on _____ and which designated the U.S.”

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

“The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectively. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application.”

☐ “The nonprovisional application designated above, namely application _____/_____, filed _____, claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:

FILING DATE

_____/_____	_____”
_____/_____	_____”
_____/_____	_____”

☐ Where more than one reference is made above please combine all references into one sentence.

18. Relate Back—35 U.S.C. 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

Country	Appln. no.	Filed
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The certified copy(ies) has (have)

☐ been filed on _____, in prior application _____, which was filed on _____.

☐ is (are) attached.

WARNING: *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may not be relied on without any need to file a certified copy of the priority application in the continuing application. This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

19. Maintenance of Copendency of Prior Application

NOTE: *The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).*

A. ☐ Extension of time in prior application

*(This item **must** be completed and the papers filed in the prior application, if the period set in the prior application has run.)*

☐ A petition, fee and response extends the term in the pending **prior** application until _____

☐ A copy of the petition filed in prior application is attached.

B. ☐ Conditional Petition for Extension of Time in Prior Application

(complete this item, if previous item not applicable)

☐ A conditional petition for extension of time is being filed in the pending **prior** application.

☐ A copy of the conditional petition filed in the prior application is attached.

20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed

(complete applicable item (a), (b) and/or (c) below)

(a) ☒ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

☒ the same.

☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

(type name(s) of inventor(s) to be deleted)

(b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

☐ the same.

☐ the following additional inventor(s) have been added:

(type name(s) of inventor(s) to be deleted)

(c) ☐ The inventorship for all the claims in this application are

☐ the same.

☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made

☐ is submitted.

☐ will be submitted.

21. Abandonment of Prior Application *(if applicable)*

☐ Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include

the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

WARNING: "The claims of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." MPEP, § 706.07(b).

NOTE: Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

(check the next item, if applicable)

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

23. Small Entity (37 CFR § 1.28(a))

☒ Applicant has established small entity status by the filing of a statement in parent application
60/064,893, filed November 7, 1997

☒ A copy of the statement previously filed is included.

WARNING: See 37 CFR § 1.28(a).

24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

☐ A notification of the filing of this
(check one of the following)

☐ continuation

☐ continuation-in-part

☐ divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

SOMATIC TRANSFER OF MODIFIED GENES TO PREDICT DRUG EFFECTS

The present application is a continuation of co-pending U.S. provisional application serial number 60/064,893, filed November 7, 1998, which is incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

5 1. Field of the Invention

The present invention relates to somatic cell gene transfer methods for mimicking one or more effects of a drug candidate compound. In one aspect, the methods mimic the effect of a drug candidate compound with potential to potentiate or suppress activity of a selected target molecule. In another aspect, the methods
10 provide means of identifying a molecular target for the drug candidate compound. The present methods have a variety of uses including providing identified molecular targets for use in drug screens.

2. Background

Eukaryotic organisms include a variety of cells, tissues and organs that react to
15 stimuli. Cell pathways have evolved to react to the stimuli. The cell pathways generally include molecules such as receptors, pore- or channel-forming proteins, enzymes, growth factors, differentiation factors, messenger molecules, immunocompatibility factors and certain structural molecules. See generally Alberts, B. et al. (1989) in *The Molecular Biology of the Cell* 2nd ed. Garland
20 Publishing, Inc. New York; and Kandel, E.R. et al. (1991) in *Principles of Neuroscience* 3rd ed. Appleton & Lange, Norwalk, Ct.

In particular, specified cell pathways include complexes of ion channel proteins that propagate charge between certain electrically responsive cells. See e.g., Hille, B. (1991) *Ionic Channels of Excitable Membranes*, 2 ed. Sunderland, Mass.
25 Sinauer.

The cell pathways play a major role in the function of eukaryotes. Accordingly, there have been substantial efforts toward identifying drugs with therapeutic capacity to modulate the cell pathways in a predictable manner.

Several traditional approaches have been developed to detect drugs with therapeutic capacity to modulate the cell pathways.

For example, "natural products testing" is one such approach. In a typical approach, material is obtained from a biological source and then screened for a pre-determined activity. Positive results are usually followed by purification of the material from the biological source and identification of the molecule(s) responsible for the activity. See e.g., Hardman et al. (1996) eds. in *Pharmacological Basis of Therapeutics* 9th ed. McGraw-Hill.

Other approaches encompass what is sometimes referred to as "synthetic chemical testing". In this approach, a promising lead compound is synthesized and optionally derivatized by specified chemical manipulations. Typically, there exists substantial knowledge with respect to the structure and function of that lead compound as well as the manipulations necessary to develop a drug candidate compound from the lead compound. See e.g., Hardman et al. *supra*.

However, such traditional approaches for developing drugs have recognized shortcomings.

For example, natural products testing can require access to a large variety of organisms many of which may be rare or produce drug candidate compounds in limiting quantities. Labor and time investments are typically substantial. In most cases, there is incomplete knowledge as to the structure and function of the compounds. Even less is usually known about the molecular targets of such compounds.

Approaches relying on synthetic chemical testing have suffered from related shortcomings. For example, chemical manipulations required to make a drug candidate compound can be labor intensive and require several years of research effort. In many instances, development of a promising compound has proceeded without adequate knowledge of the molecular target(s) of that compound.

More generally, the traditional approaches have been hampered by inadequate knowledge of the molecular targets of many drug candidate compounds. That lack of knowledge can negatively impact efforts to identify and develop the compounds. By

focussing on the target molecule of the compound, the traditional approaches may miss opportunities to detect a variety of promising compounds.

In particular, a promising drug candidate compound may go undetected in a traditional approach if that compound cannot interface with a molecular target. For example, a compound with unsuitable solubility or stability may be discarded in a traditional screen even though that compound can potentiate or suppress activity of a key target molecule such as a protein.

There have been recent attempts to circumvent or at least reduce the need to identify the molecular targets of drug candidate compounds. In general, screens have been implemented that substantially increase the diversity of known lead compounds. The objective has been to increase the number of lead compounds in the hope of identifying a compound of interest.

For example, combinatorial chemistry is one approach for substantially increasing the diversity of lead compounds. The technique is premised on the general assumption that production of large libraries of lead compounds and particularly lead compound derivatives, will provide a pool from which can be selected a significant number of drug candidate compounds. See e.g., Brenner et al. (1992) *PNAS (USA)* 89: 5381 Nielsen et al. (1993) *J. Am. Chem. Soc.* 115: 9812 and references cited therein.

More recently, there have been efforts to integrate computer-assisted modeling techniques and combinatorial chemistry into a unified screening approach for detecting drug candidate compounds. The approach is sometimes referred to as "targeted diversity" or "rational drug design".

However, combinatorial chemistry and targeted diversity suffer from significant drawbacks. For example, although these approaches have potential to increase pools of drug candidate compounds, implementation is not always cost and time effective. For example, these screening techniques are often negatively impacted by a lack of understanding of molecular targets of the lead compounds.

Accordingly, in many instances the molecular targets of the drug candidate compounds are unknown, thereby potentially stalling development of future compounds. Efforts to improve existing compounds may suffer a related fate.

There has been substantial research efforts toward identifying ion channels
5 such as potassium channels. The ion channels represent a highly diverse family of proteins of particular pharmacological interest. The proteins are known to form multimeric membrane complexes. See e.g., Deal, K.D. et al. (1996) *Phys. Rev.* 76: 49; Rudy, B. (1998) *Neuroscience* 25: 729.

Specifically, certain ion channel proteins have been reported to be involved in
10 human diseases such as epilepsy, heart failure and inherited long QT syndrome. See e.g., Bassett, A. L., et al. (1994) *Circ Res* 75:296; Tsaur, M. L., et al. (1992) *Neuron* 8: 1055; Kaab, S., et al. (1996) *Circ Res* 78(2), 262-73; and Good, T. A., et al. (1996) *Biophys. Journal* 70: 296.

Selective gene suppression is an approach for understanding protein function.
15 For example, antisense and dominant negative methodologies have been used to manipulate function of certain ion channel proteins. See e.g., Wagner, R.W. (1995) *Nature Medicine* 1: 1116.

It thus would be useful have assays that mimic the effects of drug candidate compounds and particularly those compounds with capacity to potentiate or suppress
20 activity of a specified target molecule. It would be particularly desirable to have *in vitro* assays that can identify the target molecule without complete knowledge of the compound. It would be further desirable to have *in vitro* assays that effectively mimic diseases or disorders impacted by the target molecule and the compound.

SUMMARY OF THE INVENTION

25 The present invention provides novel somatic gene transfer methods for mimicking one or more effects of a drug candidate compound. Generally stated, the methods are *in vitro* assays that modulate expression of a selected target molecule and analyze consequences of that modulation to evaluate effects of the drug candidate compound. Preferred methods of the invention positively identify target molecules
30 for drug candidate compounds with capacity to potentiate or suppress activity of that

target molecule. Particularly preferred methods mimic human diseases or disorders impacted by the target molecule and the drug candidate compound.

Significantly, the present methods are complementary to traditional drug screening approaches and can enhance those approaches by providing identified target
5 molecules, particularly in the absence of lead compounds.

Preferred methods of the present invention are *in vitro* assays that involve somatic cell gene transfer and include one or more of the following steps 1) through 4):

- 1) culturing in medium a population of selected somatic cells, a tissue or
10 an organ capable of expressing a recombinant nucleic acid segment such as a protein, preferably a native protein;
- 2) modifying the nucleic acid segment sufficient to modulate the result of expression of the protein in the cells, tissue or organ;
- 3) introducing the modified nucleic acid segment into the cells, tissue or
15 organ under somatic cell gene transfer conditions sufficient to express a modified protein; and
- 4) analyzing the result of expression of the modified protein to mimic (or predict) the effect of the drug candidate compound in the cells, tissue or organ.

The result of expression of the modified protein mimics one or more effects of
20 the drug candidate compound, particularly those compounds with capacity to potentiate or suppress activity of the protein expressed by the nucleic acid segment.

Particularly, step 4) above can encompass correlating the result of expression of the modified protein to the result of expression of a corresponding native protein. Sometimes, that native protein will be referred to herein as a "full-length" "normal" or
25 "wild-type" to denote natural occurrence of that native protein in the selected somatic cell, tissue or organ. Analysis of the result of expression of the native protein can be conducted in a parallel (i.e. control) assay that includes one or more of the steps 1) through 4) above except that the protein is not modified.

A modified protein of the present invention typically exhibits at least 10
30 percent or greater activity relative to the full-length protein; more preferably at least

20 percent or greater activity; and still more preferably at least about 30, 40, 50, 60, 70, 80, 100, 150, or 200 percent or greater activity relative to the full-length protein. Such activity of the modified protein will be considered indicative of mimicking activity of a drug candidate compound. Particularly preferred are those activities that point to drug candidate compounds which would potentiate (i.e. greater than 100%) or suppress (i.e. less than 100%) activity of the corresponding native protein in the selected cell, tissue or organ.

Thus, "mimicking" as the term is used herein is evidenced by an activity of the modified protein in the above generally described assay when compared to the native protein (i.e. control). That activity typically manifests a detectable phenotype in the selected somatic cell, tissue or organ. For example, the phenotype can be a morphological alteration or a functional change that can be assayed (and quantitated if desired) by a method known in the art including those specified below.

Significantly, the change in activity of the modified protein can be impacted by several factors including 1) the somatic cell, tissue or organ selected, 2) the target molecule selected and particularly the specificity the drug candidate compound would have for that molecule, and 3) the potency of the drug candidate compound would have in that selected cell, tissue or organ.

More particularly, by the term "mimic" or "predict" as those terms relate to invention, is meant an identified consequence the drug candidate compound would have in the selected cell, tissue or organ. That consequence is manifested by expression of the modified protein when that expression is compared to its native counterpart. Thus, by mimicking the effect of a drug candidate compound, particularly compounds that would potentiate or suppress activity of the selected target molecule, the present methods can be performed without knowledge the drug candidate compound.

For example, the present invention does not require knowledge of the structure or synthesis of the drug candidate compound in order to identify the molecular target of that compound.

Molecular targets and particularly proteins targets such as ion channels identified in accord with the invention will generally find important use, e.g., as important starting reagents in traditional drug discovery screens. Such targets will be particularly useful in those traditional drug screens for which there is no reliable lead
5 compound.

Reference herein to a "standard *in vitro* assay" in accord with the invention refers to the above protocol of steps 1) through 4).

A nucleic acid segment suitable for use with the present invention can be nearly any nucleic acid segment that encodes a protein such as a cDNA or genomic
10 sequence. Preferably the encoded protein is full-length although in some instances the nucleic acid segment may encode a protein fragment.

It is particularly preferred that the nucleic acid segment be compatible with conventional somatic cell gene transfer techniques such as described herein.

Somatic cell gene transfer techniques in accord with the invention generally
15 include standard techniques for controllably expressing an introduced gene into non-germ line cells (i.e. non-sperm or egg cells). For example, in one approach, what is often referred to as "*ex vivo*" methods are used to remove somatic cells from a host organism (sometimes known as primary cells). The cells are then used as recipient cells for transfer of a gene of interest into the cells. Subsequently, the genetically
20 modified cells can be implanted back into the recipient host organism. Particularly, such techniques can be use for study of cells *ex vivo*, e.g., in the phenotypic characterization of ion channel protein "knock-outs".

In what is sometimes referred to as an "*in vivo*" somatic cell gene transfer technique, a desired gene is introduced into cells of a host organism without removing
25 those cells from the host organism.

Preferred methods of the invention typically employ *ex vivo* somatic cell gene transfer or a related *ex vivo* technique to introduce a nucleic acid segment of interest into selected somatic cells, tissues or organs. Typically, use of that technique is sufficient to express the protein encoded by nucleic acid segment in quantities
30 detectable at the RNA or protein level.

The present methods can be performed with nearly any cell, tissue or organ that is compatible with a recognized somatic cell gene transfer techniques and particularly those techniques that include *ex vivo* manipulations. Exemplary cells include primary or native cells obtained directly from a host animal of interest such as a mammal and particularly a primate such as a human. More particularly, the cells are somatic in the sense that the cells are not germ-line cells and can include such cells as those obtained from the nervous or circulatory system, the immune, reproductive, digestive, respiratory or lymphatic system, the hepatic system, or the skin. Particular examples include cells isolated from heart tissue. As noted, groups of such cells may also be used in the form of selected tissues or organs.

More specifically, a somatic cell, tissue or organ of interest is compatible with a recognized somatic cell gene transfer technique and particularly the *ex vivo* technique outlined above, if at least 20%, preferably at least 50%, 60%, 70%, and more preferably at least 95% or more of the total number of cells are transformed by use of technique. Methods for determining the percentage of cells transformed by somatic cell gene transfer technique are known in the field and include analyzing expression of a detectable reporter protein (e.g., β -galactosidase, luciferase or the like) by well known immunological methods such as those employing monoclonal antibodies and/or fluorescence microscopy.

It has been reported that certain somatic cell gene transfer techniques and particularly *ex vivo* techniques rely on viral delivery of a desired nucleic acid segment. Thus, a nucleic acid segment for use in accord with the present invention must be compatible with the chosen viral delivery system. As a specific example, for somatic cell gene transfer delivery systems using viral vectors and particularly adenovirus or adenovirus-based vectors, the nucleic acid segment will typically be ligated into a suitable site in the vector which facilitates expression in the selected cell, tissue or organ. It is preferred that the size of the nucleic acid segment will not increase the size of that vector by more than about 1% to 10 % , preferably no more than about 1% to 5%, and more preferably no more than about 1%, 2%, 3%, or 4%.

A large variety of encoded proteins are compatible with the present invention. The function of the protein can be unknown in which case the phenotypic consequences of expressing the protein can be detected by procedures known in the field. Alternatively, the function of the protein can be known or suspected in which case the result of expressing the wild-type or modified protein can be readily tested by specified assays such as those described below.

Thus, by the term "result" or "consequence" of expression is meant a phenotype that is manifested in the selected cells, tissue or organ by expression of the full-length or modified protein. The result of expression can be detected by one or a combination of different strategies such as those described below.

Preferred time frames for assaying the result of expression of a protein will vary with the protein, cell, tissue or organ chosen. However it is generally preferred that the result of expression be manifested within about 10 minutes to 72 hours or more up to about 1 to 2 weeks, preferably within about 30 minutes to 48 hours, more preferably within about 1 to 12 hours and even more preferably within about 1 to 2 hours following somatic gene transfer of the nucleic acid segment into the selected cells, tissues or organs.

For example, many phenotypic manifestations such as specified morphology changes and functional changes fall within those time frames when assayed by most somatic cell gene transfer techniques. Such phenotypes include, but are not limited to, propagation of an electrical charge, growth, blebbing or budding, pycnotic transformation, kinesis, cell death, differentiation, replication, transcription, translation, protein processing, adhesion, oncogenic transformation, enzymatic catalysis, protein modification, etc.

As noted, the present invention is compatible with use of a large variety of nucleic acid sequences and particularly cDNA sequences. A preferred cDNA encodes a protein that typically includes a signal sequence, a mature protein sequence (sometimes referred to as a complete coding sequence or "cds"); and a poly-adenylation sequence. In particular, the cds will include a single open-reading frame (ORF) and will usually also include one or more protein "motifs" e.g., one or more

transmembrane domains, extracellular and intracellular domains, ligand binding domains, and/or protein modification site such as those involved with glycosylation, phosphorylation, or protein processing site.

In general, the cDNA or other suitable nucleic acid sequence of interest is
5 inserted into a suitable somatic cell transformation vector such as those specific adenovirus vectors described below.

Significantly, most cds sequences and protein motifs are readily detectable by a variety of well-known computer-assisted programs. In particular, many of the programs can establish relationship between an unknown protein and a known protein.
10 Thus, for a nucleic acid segment and particularly a cDNA sequence encoding a protein of unknown function, a function can often be assigned, e.g., by computer-assisted programs such as those specifically identified below.

As noted above, a full-length protein encoded by a cDNA will sometimes be referred to as a normal, or wild-type protein to denote natural occurrence of that
15 protein in a subject, particularly a mammal and more particularly a primate such as a human. In contrast, a protein "modified" in accordance with the invention does not usually occur in that subject and must be made by recombinant manipulations described herein.

More specifically, a protein "modified" in accordance with the invention can,
20 e.g., be overexpressed relative to the wild-type protein.

For example, one way to overexpress the protein is to operably link a cDNA encoding that protein to a strong viral promoter such as the cytomeglovirus (CMV) or rous sarcoma (RSV) viral promoter. Following somatic gene transfer in a suitable transformation vector, that protein is typically overexpressed relative to the native
25 protein.

As will be explained more fully below, a protein modified in accord with the invention may also include one or more contiguous or non-contiguous amino acid additions, substitutions, or deletions relative to the full-length protein sequence.

As will also be explained, certain modifications of proteins in accord with the
30 invention will be genetically dominant so as to inhibit function of other proteins

particularly those proteins capable of forming binding complexes with the wild-type protein. Sometimes those modified proteins will be referred to as "dominant negative" proteins.

It will be appreciated by those of skill in the field that a protein in accord with the invention can be essentially full-length. That is, the protein may not have all the components of a full-length sequence. For example, the protein may include a complete or nearly complete cds but lack a complete signal or poly-adenylation sequence. It is preferred that a nucleic acid and particularly a cDNA encoding a protein include at least a complete cds. That cds is preferably capable of encoding a protein exhibiting a molecular weight of between about 0.5 to 70, preferably between about 5 and 60, and more preferably about 15, 20, 25, 30, 35, 40 or 50 kD. That molecular weight can be readily determined by suitable computer-assisted programs or by SDS-PAGE gel electrophoresis.

Although generally not preferred, the nucleic acid segment can be a genomic sequence or fragment thereof comprising one or more exon sequences. In this instance it is preferred that the cell, tissue or organ selected for performing somatic cell gene transfer be capable of correctly splicing any exon sequences so that a full-length or modified protein can be expressed.

The nucleic acid segment and particularly the cDNA encoding the full-length protein can be modified by conventional recombinant approaches to modulate expression of that protein in the selected cells, tissues or organs.

More specifically, the nucleic acid segment can be modified by recombinant methods that can add, substitute or delete one or more contiguous or non-contiguous amino acids from that encoded protein. In general, the type of modification conducted will relate to the result of expression desired.

For example, a cDNA encoding a protein of interest such as an ion channel can be modified so as overexpress that protein relative to expression of the full-length protein (i.e. control assay). Typically, the modified protein will exhibit at least 10 percent or greater overexpression relative to the full-length protein; more preferably at

least 20 percent or greater; and still more preferably at least about 30, 40, 50, 60, 70, 80, 100, 150, or 200 percent or greater overexpression relative to the control assay.

As noted above, further contemplated modifications to a nucleic acid segment and particularly a cDNA are those which create dominant negative proteins.

5 In general, a variety of dominant negative proteins can be made by methods known in the field. For example, ion channel proteins are recognized as one protein family for which dominant negative proteins can be readily made, e.g., by removing selected transmembrane domains. In most cases, the function of the ion channel binding complex is substantially reduced or eliminated by interaction of a dominant
10 negative ion channel protein.

Several specific strategies have been developed to make dominant negative proteins. Exemplary of such strategies include oligonucleotide directed and targeted deletion of cDNA sequence encoding the desired protein. Less preferred methods include nucleolytic digestion or chemical mutagenesis of the cDNA.

15 It is stressed that creation of a dominant negative protein is not synonymous with other conventional methods of gene manipulation such as gene deletion and antisense RNA. What is meant by "dominant negative" is specifically what is sometimes referred to as a "poison pill" which can be driven (i.e. expressed) by an appropriate DNA construct to produce a dominant negative protein which has capacity
20 to inactivate an endogenous protein.

For example, in one approach, a cDNA encoding a protein comprising one or more transmembrane domains is modified so that at least 1 and preferably 2, 3, 4, 5, 6 or more of the transmembrane domains are eliminated. Preferably, the resulting modified protein forms a binding complex with at least one other protein and usually
25 more than one other protein. As noted, the modified protein will inhibit normal function of the binding complex as assayed, e.g., by standard ligand binding assays or electrophysiological assays. Exemplary binding complexes are those which participate in electrical charge propagation such as those occurring in ion channel protein complexes. Typically, a dominant negative protein will exhibit at least 10
30 percent or greater inhibition of the activity of the binding complex; more preferably at

least 20 percent or greater; and still more preferably at least about 30, 40, 50, 60, 70, 80, or 100 percent or greater inhibition of the binding complex activity relative to the full-length protein.

As a further illustration, a cDNA encoding a desired protein for use in the present methods can be modified so that at least one amino acid of the protein is deleted. The deleted amino acid(s) can be contiguous or non-contiguous deletions essentially up to about 1%, more preferably about 5%, and even more preferably about 10, 20, 30, 40, 50, 60, 70, 80, or 95% of the length of the full-length protein sequence.

Alternatively, the cDNA encoding the desired protein can be modified so that at least one amino acid in the encoded protein is substituted by a conservative or non-conservative amino acid. For example, a tyrosine amino acid substituted with a phenylalanine would be an example of a conservative amino acid substitution, whereas an arginine replaced with an alanine would represent a non-conservative amino acid substitution. The substituted amino acids can be contiguous or non-contiguous substitutions essentially up to about 1%, more preferably about 5%, and even more preferably about 10, 20, 30, 40, 50, 60, 70, 80, or 95% of the length of the full-length protein sequence.

Although generally less-preferred, the nucleic acid segment encoding the desired protein can be modified so that at least one amino acid is added to the encoded protein. Preferably, an amino acid addition does not change the ORF of the cds. Typically, about 1 to 50 amino acids will be added to the encoded protein, preferably about 1 to 25 amino acids, and more preferably about 2 to 10 amino acids. Particularly preferred addition sites are at the C- or N-terminus of the selected protein.

The present invention also provides a method of detecting an ion channel capable of serving as a drug target protein, the method comprising the following general steps 1) through 5):

- 1) providing a population of somatic cells capable of producing an ion current from a recombinant nucleic acid segment encoding an ion channel protein;

- 2) modifying the nucleic acid segment sufficient to alter the ion current produced by the encoded ion channel protein;
- 3) transferring the modified nucleic acid segment into the somatic cells under somatic cell gene transfer conditions which allow expression of the encoded ion channel and production of an altered ion current;
- 4) detecting the altered ion current; and
- 5) correlating the altered ion current to the capacity of the ion channel protein to serve as the drug target protein.

The expression of the ion channel protein can be increased or inhibited (e.g., by truncation) following the somatic gene transfer relative to a corresponding native gene. For example, the truncation can be a contiguous or non-contiguous deletion of the transferred gene. Alternatively, expression of the target protein can be inhibited by transfer of a gene encoding a modified protein comprising one or more amino acid substitutions relative to a corresponding native protein.

As noted, some of the target proteins of the method can form a binding complex with at least one other protein as in the case of certain ion channel proteins. For example, expression of the target protein in the method can be sufficient to produce a dominant negative protein that reduces or blocks function of the binding complex.

The present invention also features a method of reproducing human disease, e.g., diseases of electrically excitable cells such as cardiac cells and particularly a cardiac arrhythmia phenotype. In general, the method includes the following general steps 1) to 5):

- 1) providing a population of cultured somatic cells capable of producing an ion current from a recombinant nucleic acid segment encoding a selected ion channel protein;
- 2) modifying the nucleic acid segment sufficient to alter the ion current produced by the encoded ion channel protein;

- 3) transferring the modified nucleic acid segment into the cells under somatic cell gene transfer conditions which allow expression of the encoded ion channel and production of the altered ion current;
- 4) detecting the altered ion current; and
- 5) correlating the altered ion current to the capacity of the ion channel to serve as the therapeutic target. Typically, that correlation will include comparing the altered ion current to that ion current produced by the full-length protein (i.e. control assay).

The invention further provides a method of reproducing a cardiac arrhythmia phenotype in a population of cultured cells, the method comprising the following general steps 1) to 5):

- 1) providing a population of cultured somatic cells capable of producing an ion current from a recombinant nucleic acid segment encoding an ion channel protein;
- 2) modifying the nucleic acid segment sufficient to alter the ion current produced by the encoded ion channel protein;
- 3) transferring the modified nucleic acid segment into the cells under conditions which allow expression of the encoded ion channel and production of the altered ion current; and
- 4) detecting the altered ion current sufficient to reproduce the mammalian cardiac arrhythmia in the cultured cells.

Preferred methods include modifying the nucleic acid segments sufficient to overexpress the ion channel protein in the population of somatic cells. Further preferred are method in which the nucleic acids segments are modified to produce a dominant negative ion channel protein. The ion channel protein can be, e.g., a sodium, calcium, voltage-gated, or ligand-gated ion channel and particularly a potassium ion channel.

Disclosure relating to such channel proteins can be found in the discussion above and in U.S. Pat. No. 5,436,128 to Harpold, M.M et al.

Further provided by the present invention is a method of mimicking one or more effects of a drug candidate compound in an identified somatic cell, tissue or organ of interest, the method comprising the following general steps 1) and 2):

- 1) modulating, by somatic gene transfer, expression of a selected protein
5 in selected cells; and
- 2) analyzing the result of expression of the protein to thereby predict the effect of the drug candidate compound.

Preferably the drug candidate compound would increase or suppress activity of the protein in the cell, tissue or organ. The method can further include use of a
10 traditional drug discovery strategy such as those mentioned above and including natural products testing, synthetic chemical testing, etc., and use of the protein identified by the method.

As mentioned in the previous discussion, the present invention has significant uses.

15 For example, the methods are capable of identifying molecular targets of drug candidate compounds that would potentiate or suppress activity of those targets. The molecular targets include proteins which sometimes exist, e.g., as glycoproteins, lipoproteins, glycolipoproteins, or phosphorylated proteins. Alternatively, or in addition, the targets may be complexed to form a binding complex. Thus, the present
20 invention can be used to identify molecular targets as they naturally occur inside selected cells, tissue or an organ.

The present invention can thus be used to select molecular targets suitable for testing, e.g., in traditional drug discovery methods such as those highlighted above.

25 Significantly, the present invention is complementary to traditional drug discovery methods and potentially enhances those methods by providing selected molecular targets, particularly in the absence of lead compounds.

Further contemplated uses of the present invention include assessment of the effects of existing drugs on selected somatic cells, tissues and organs that have been genetically modified to reproduce or mimic a human disease. The present invention
30 can also be used as a research or medical tool to investigate the functional role of

specified gene products in a desired cell, tissue, or organ. In addition, the present invention can be used to create animal models of human disease, e.g., by implanting the genetically modified cells into a suitable recipient in accord with the *in vivo* techniques mentioned above.

- 5 Additionally, the present methods can be combined with traditional drug discovery programs including the traditional screens outlined above to provide a comprehensive and rational drug screening program in which molecular targets are positively identified.

- 10 The present invention provides a number of significant advantages. For example, by identifying molecular targets of the drug candidate compounds, the methods can provide verification of a proposed or suspected molecular target. Alternatively, that verification can be advantageous toward the goal of understanding how an existing drug functions. As noted, such fundamental knowledge can be used as a starting point for implementation of a traditional drug discovery effort.
- 15 Alternatively, that knowledge can also be used to focus an ongoing drug screen to identify those drug candidate compounds with likelihood of potentiating or suppressing an identified molecular target.

- 20 The present methods provide other significant advantages. For example, preferred assays of the invention employ living somatic cells, tissue or organs as screening media, thereby allowing molecular targets to be identified in a physiologically relevant setting.

- 25 Significantly, the present methods purposefully engage in the selection of molecular targets in the context of a desired cell, tissue or target tissue. In contrast, traditional drug screens often employ reconstituted or heterologous systems in well-studied model cells such as *Xenopus* or immortalized tissue culture cells. As noted, the present methods are not limited to these cells. Instead, the present methods can be used with nearly any cell, tissue or organ that is compatible with the somatic cell gene transfer techniques as discussed above.

- 30 The present methods provide even further advantages. For example, prior practice often required introduction of a cDNA encoding a modified protein of interest

into the germ line of certain animals. The resulting transgenic animals usually have the nucleic acid segment incorporated into chromosomes (or deleted therefrom in the case of a "knock-out" mutation).

In contrast, the present methods differ from conventional germ line transformation in that the methods use somatic gene transfer to achieve targeted expression of a desired protein. Thus, the present methods are compatible with *de novo* modification of isolated cells, tissues or organs. As noted above, the effects of a nucleic acid segment transferred in accord with the invention are fully manifested usually between several minutes up to one or two days or longer in culture. It is believed that in this setting, the result of expression of a modified protein more closely reproduces or mimics consequences of acute drug exposure. Approaches relying on germ line manipulations are believed to be particularly incompatible with that objective.

Significantly, the present methods are highly versatile and are compatible with analysis in variety of somatic cells, tissues, and organs. In particular, the present methods are relatively inexpensive to perform, and can be conducted in most laboratory settings.

Further advantages are provided by the present invention. For example, the methods support ongoing medical and research efforts that seek to understand known or suspected principles of drug action. As will become more apparent from the discussion and examples which follow, those principles can be readily testing by the present methods. Importantly, proteins modified and expressed in accord with the invention have highly specific functional alternations that reproduce or mimic potential drug actions in the selected somatic cell, tissue or organ.

Other aspects of the invention are discussed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-E are references to illustrative cDNA sequences that can be used in accordance with the present invention. Additional cDNA sequences are referenced in the text.

Figures 2A-F are graphs illustrating canine LV myocytes infected with AdGFP or AdHERG.

Figures 3A is a graphical illustration of the KV4.2FL and KV4.2ST ion channel proteins.

5 Figures 3B-C show the amino acid sequence of the Kv4.2Fl and Kv4.2ST proteins.

Figures 4A-B are schematic representations of the Kv4.2 (4A) and Kv4.2ST (4B) proteins.

Figure 5 is a map of the E1-KV4.2ST vector.

10 Figure 6 is a map of the pJM17 vector.

Figures 7A outlines a recombination strategy for making the KV4.2ST vector.

Figure 7B depicts the map of the Ad4.2ST sequence resulting from the strategy outlined in Figure 7A.

15 Figure 8 is a representation of an RNase protection assay blot showing that AdKv4.2ST expresses only the sense strand of the Kv4.2ST sequence.

Figures 9A-F are graphs showing specific suppression of Kv4.2 currents by pE1Kv4.2ST.

Figures 10A-B are graphs showing whole-currents of KV4.2ST.

20 Figures 11A-B are graphs showing whole-currents of Ad-KV4.XST in canine ventricular myocytes.

Figures 12A-C are graphs showing that AdKv4.2St suppresses the A-type current of cerebellular granule neurons.

Figures 13A-C are graphs depicting suppression of I_{tol} by AdKv4.2ST in adult rat ventricular myocytes.

25 Figures 14A-I are graphs showing that modified Kv4.2ST (GFP-Kv4.2ST) specifically suppresses *Shal* protein family currents.

Figures 15A-B are confocal photomicrographs illustrating GFP-Kv4.2ST protein distributed in various cell membranes.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, the present invention features methods and particularly *in vitro* assays for mimicking one or more effects of a drug candidate compound.

5 The ability to mimic the effects of a drug candidate compound and to identify the molecular target of that compound has been found to be very important. For example, it is believed that traditional drug screening approaches including those mentioned above may sometimes miss identification of promising drug candidates.

10 Particularly, several parameters related to performing traditional screens may negatively impact identification of the compounds, e.g., pleotropic effects, toxicity, or synergy manifested by the compound. In addition activity may be hidden by problems relating to solubility, permeability, stability or metabolism of the drug candidate compound in those screens.

15 In contrast, the present methods overcome many of these problems by predicting the effects of a drug candidate compound without complete knowledge of that compound and particularly without knowing the structure or synthesis of the compound.

In general, the present invention involves use of conventional techniques such as those relating to genetics, electrophysiology and recombinant DNA technology.

20 In particular, preparation of the nucleic acids disclosed herein involves standard recombinant manipulations, e.g., polymerase chain amplification reactions (PCR), preparation of plasmid DNA, cleavage of DNA with restriction enzymes, ligation of DNA, isolation of mRNA, introduction of the DNA into a suitable cell, culturing of the cell, SDS-PAGE gel electrophoresis, RNase protection assays, immunological techniques such as RIA, ELISA and Western Blot hybridization, 25 Northern blots, scintillation coating, densitometry, construction of adenovirus vectors and autoradiography. See generally, Sambrook et al., *Molecular Cloning: A Laboratory Manual*. (2nd ed. (1989); Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1989), for discussion relating to many of the standard methods, the disclosures of which are incorporated herein by reference.

Further, methods for conducting electrophysiological experiments described herein include voltage-clamp type assays and measurement of whole currents. See e.g., Hille, B. *supra*; Miller C. (1987) *Neuromodulation: The Biological Control of Neuronal Excitability*. L.K. Kaczmark and I.B. Levitan (eds.) New York: Oxford University Press; and Armstrong, C.M. (1981) *Physiol. Rev.* 61: 644, the disclosures of which are incorporated by reference .

As noted, the present methods are *in vitro* assays that involve predicting the effect of a drug candidate compound, modulating, by somatic gene transfer, expression of a selected target protein in selected cells, and analyzing the result of expression of the target protein to predict the effect of the drug candidate compound.

As also noted, a nucleic acid segment suitable for use with the present methods is preferably a cDNA sequence encoding a full-length (or modified) protein. Typically, the cDNA will be formatted as a DNA vector appropriate for a somatic cell gene transfer technique as described herein. Preferred vectors are viral DNA vectors and particularly adenovirus vectors adapted for use with the techniques. Preferred adenovirus vectors and methods for performing somatic cell gene transfer have been described.

A cDNA suitable for use in accord with the invention can be obtained from a variety of public and commercial sources.

For example, one source is the National Center for Biotechnology Information (NCBI)- Genetic Sequence Data Bank (Genbank). A DNA sequence listing can be obtained from Genbank at the National Library of Medicine, 38A, 8N05, Rockville Pike, Bethesda, MD 20894. Genbank is also available on the internet at <http://www.ncbi.nlm.nih.gov>. See generally Benson, D.A. et al. (1997) *Nucl. Acids. Res.* 25: 1 for a description of Genbank.

More particularly, Genbank provides a variety of cDNA sequences suitable for use with the present invention. Selection of a suitable cDNA from Genbank or another appropriate source of cDNAs will be guided, e.g., by the type of molecular target desired (i.e. enzyme, ion channel, etc.), the somatic cells, tissues or organ with

which somatic cell gene transfer is to be performed, and the mode of viral gene delivery selected.

Figure 1 below provides an illustrative list of cDNA sequences obtained from the Genbank database that can be used with the present methods. The figure lists
5 proteins encoded by the cDNA sequences under "Description" and cites reference designations under "Reference" and "mRNA/gene sequence" in accord with nomenclature adopted by Genbank. The cDNA sequences can be readily accessed on-line by using the designations provided by Genbank.

For example, referring again to Figure 1, a suitable cDNA sequence is the
10 potassium channel beta 1a subunit. That cDNA can be accessed on-line by referencing the designation U33428. In addition, other reference designations are provided in the figure for that channel subunit.

See also the examples which follow for an illustrative use of the KvS4.2FL potassium channel cDNA sequence.

15 It is possible to obtain cDNA sequences from Genbank by one or a combination of alternative approaches.

For example, one approach is to perform a routine PCR experiment based on the provided cDNA sequence information.

In a specific approach, a complimentary pair of opposing oligonucleotide
20 primers can be made that are spaced between about 100 to 5000 kb apart, preferably about 200, 300, 400, 500, 700, 800, 900 to 4000 kb apart, and more preferably about 1000 kb apart depending, e.g. on the size of the selected cDNA. The cDNA can be amplified and purified according to standard methods. The amplified DNA is then inserted into a somatic cell gene transfer vector such as preferred adenovirus vectors
25 described below for somatic cell gene transfer. Typically, the cDNA will be ligated to a suitable oligonucleotide linker (i.e. a polylinker) at each end of the cDNA so that the nucleic acid can be ligated to the vector with one or more known restriction sites. See the examples which follow.

As mentioned previously, the present invention can be used with a nucleic acid segment and particularly a cDNA that encodes a protein of known or unknown function.

For example, if the cDNA encodes a protein of unknown function, it is possible to determine phenotypic consequences of overexpressing or making a dominant negative of that protein by strategies known in the field.

For example, one such strategy is to assign a function to that unknown protein. That can be achieved by determining homology between the unknown protein and one or more other proteins of known function. That assignment is usually based on amino acid sequence homology to another protein of known or suspected function.

It is generally recognized that a significant majority of unknown proteins can be assigned a function by that strategy.

Two or more proteins are "homologous" to each other if the amino acid sequence of each protein shares at least 25% , more preferably at least 40%, and even more preferably at least 50% or more amino acid sequence identity over at least 100 amino acids after performing what is known in the field as amino acid gapping. A variety of standard computer-assisted programs are available for conducting protein homology determinations and function assignments such as those specifically referenced below.

In particular, the following well-known computer algorithms can be used to assign function to an unknown protein: FASTA, TFASTA and BLAST. Those computer algorithms and instructions for use thereof are available at the NCBI. Additional useful computer programs generally available in the field include the Chou-Fasman algorithm and the algorithm reported by Kyte and Doolittle. See e.g., Kyte, J. and R.F. Doolittle (1982) *J. Mol. Biol.* 157: 105; Chou, P.Y and G.D. Fasman (1974) *Biochemistry* 13: 222.

In many instances, it will be useful to conceptually translate a cDNA of interest and particularly a cDNA encoding an unknown protein, with the TFASTA, FASTA or BLAST algorithms. As is well-known, these algorithms can identify a variety of motifs in the encoded protein, particularly transmembrane, extracellular and

intracellular domains, signal sequences and poly-adenylation sequences. The algorithms often include standard hydropathy plot analysis to identify the transmembrane domain and other domains of interest.

Thus, in instances where it is desirable to make a dominant negative of a membrane protein and particularly an ion channel protein, it will be useful to map the transmembrane domains. That map will facilitate deletion of selected transmembrane domains to create a desired dominant negative mutation.

Alternatively, the transmembrane domains of many membrane proteins and particularly ion channel proteins are known and can be deleted in accordance with the discussion above and the examples which follow to make a desired dominant negative protein.

A cDNA of interest that has been modified to encode a dominant negative protein can be a dominant negative membrane protein such as an ion channel protein. That encoded protein can be readily tested by a variety of recognized electrophysiological methods including standard analysis in *Xenopus* oocytes. In general, those oocytes are not usually electrically responsive but they can be made so by introduction of specified ion channel proteins in accord with standard methods. Preferred cDNA constructs encode a dominant negative ion channel in which at least one transmembrane domain has been deleted up to the penultimate number of transmembrane domains in the corresponding native protein. See e.g., Hille, supra and the examples which follow for conducting assays to detect dominant negative ion channel mutations and in particular, patch-clamp type and whole current assays to accomplish same.

Dominant negative approaches have been used to map levels of specific ion channel proteins in neurons from *Xenopus*. See Ribera, A.B. (1996) *J. of Neuroscience* 16: 1123.

More specifically, in the examples below, a strategy is outlined a strategy for making and testing a dominant negative mutation of the KsV4.2 potassium channel.

As noted above, a nucleic acid segment and particularly a cDNA can encode a protein of known function. A variety of recognized tests are available for assaying

the function (or functions) of known proteins. Exemplary of such tests include, but are not limited to, recognized tests that can detect cell proliferation, growth, or differentiation; tests for detecting replication, transcription, translation, protein processing including degradation, tests for cell death including apoptosis and necrosis, cell viability tests; cell adhesion tests; kinesis and particularly pathfinding tests; neovascularization tests, oncogenic transformation tests; tests to detect small molecules such as cytokines, cyclic nucleotides, or hormones; enzyme tests; and receptor-ligand binding tests.

Additional recognized tests are as follows. As noted above, the tests are usually conducted following somatic cell gene transfer and expression of the protein of interest.

For example, one recognized test is a standard cell proliferation assay.

In particular, a selected cDNA can be modified as described above to encode a modified protein of interest. For example the encoded protein could be an enzyme which can modulate, e.g., inhibit, cell proliferation by at least 25%, preferably at least 50%, or more relative to a suitable control assay. Exemplary cell proliferation assays include counting viable cells and monitoring activity of specified citric acid cycle enzymes such as lactate dehydrogenase. A preferred assay measures incorporation of one or more detectably-labelled nucleosides into DNA, e.g., by:

- a) culturing cells transformed by somatic cell gene transfer in medium and adding) a radiolabelled nucleoside such as ^3H - thymidine in an amount between about 0.1 to 100 μCi ;
- b) incubating the cells for about 6-48 hours followed by washing; and
- c) measuring incorporation of the ^3H - thymidine into DNA over that time relative to a control culture that is prepared and incubated under the same conditions as the assay culture but does not include the potential GalT-2 modulating compound. The measurement can be achieved by several methods including trichloroacetic acid (TCA) precipitation of labeled DNA on filters followed by scintillation counting. See e.g., Chatterjee, S., *Biochem. Biophys.*

Res Comm. (1991) 181:554 and references cited therein for disclosure relating to this assay.

Additional examples of recognized cell proliferation assays employ cells of the circulation system, e.g., smooth muscle cells and particularly myocytes prepared according to standard methods. See e.g., Ross, R. *J. Cell. Biol.* (1971) 50:172; Chatterjee, S. et al. (1982) *Eur. J. Biochem.* 120:435; Bergmeyer, H.V. *In Principles of Enzymatic Analysis.* (1978) Verlag Chemie, NY; and Hilal-Dandan, R., and Khairallah, P. A. (1991) *J Mol Cell Cardiol* 23, 705.

Another suitable assay is a cell adhesion assay. In this case, a cDNA is chosen and modified as described. The modified protein, e.g., a membrane protein, preferably modulates, e.g., inhibits, cell adhesion by at least 25%, preferably at least 50%, or more relative to a suitable control assay. For example, a preferred cell adhesion assay is achieved by:

- a) labeling a first population of cells, particularly immune cells such as certain leukocytes, with a detectable label which can be a chromatic, radioactive, luminescent (e.g., fluorescent, or phosphorescent), or enzymatic label capable of producing a detectable label,
- b) contacting the first population of cells with a second population of cells such as certain endothelial cells associated with vascular intima, which cells have been detectably-labelled with a chromatic, radioactive, luminescent (e.g., fluorescent, phosphorescent), or enzymatic label preferably different from the label employed in step a); and
- c) detecting any adhesion between the first and second population of cells. The detection can be achieved by a variety of methods such as microscopy, particularly fluorescence-based photomicroscopy involving FACS; automated cell sorting techniques, immunological methods such as ELISA, RIA; and scintillation counting.

Additional assays include standard oncogenic transformation assays that include monitoring growth in liquid or semi-solid media. The assay is based on recognition that most eukaryotic cells exhibit a phenomenon known as contact

inhibition. However, transformed cells lose that contact inhibition and can grow in liquid or semi-solid growth media in a transformed (malignant) state. Preferably, the modified protein encoded by the nucleic acid segment modulates, e.g., inhibits, oncogenic transformation by at least 25%, preferably at least 50%, or more relative to a suitable control assay.

In addition, a variety of molecular assays can be employed to determine phenotypic consequences of expressing a modified protein in accordance with the invention.

For example, Northern blot assays can be performed to quantitate expression of a particular modified protein relative to a suitable control assay. Oligonucleotide primers can be readily made from the cDNA sequences and used as probes to perform the Northern blots. Generally, the oligonucleotide primers will be between 12 and 50 nucleotides in length, preferably between about 15 and 20 or 25 nucleotides in length. Alternatively, the oligonucleotide primers can be used to perform Polymerase Chain Reaction (PCR) amplification of a desired cDNA sequence (using, e.g., genomic template DNA or preferably a commercially available cDNA library known to include the cDNA) and that amplified cDNA can be used to perform the Northern blot assay. Preferred Northern blot assays involve quantitating mRNA and correlating the amount of mRNA expressed in cells, tissues or organs with the amount of mRNA produced by a full-length cDNA sequence (control). Particularly preferred assays include testing for RNase protection such as described in the examples below.

Further, a variety of immunological methods can be employed to determine the phenotypic consequence of expressing the modified protein. For example, the modified can be quantitated by a standard immunological methods, e.g., ELISA, Western blot, or RIA. Suitable antibodies and particularly monoclonal antibodies for use in the methods can be obtained from Linscott's Directory (40 Glen Drive, Mill Valley California 94941), and by the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, Md 20852.

Additional specified tests can be found in the references cited in Figure. 1. Those references disclose tests for assaying a variety of proteins including certain ion

channel proteins including specified calcium, sodium, potassium, voltage-gated, and ligand gated channels, certain growth factors and enzymes, particular onocogens and proto-oncogenes, and cell death proteins.

See also Hille, B., *supra*, Kaczmark and Levitan, *supra*; and Armstrong, C.M.
5 *supra* for general disclosure relating to tests for to ion channel proteins. See further the examples which follow for specific electrophysiological tests relating to a potassium channel protein.

As noted, preferred tests include electrophysiological assays in which a cDNA
10 encoding a modified protein is introduced into selected cells, tissues or an organ by somatic cell gene transfer. Typically, the cells are electrically responsive, but as mentioned above the cells may include certain oocytes that are not usually responsive to charge. Following transfer of the cDNA into the cells, tissue or organ, whole-currents are measured with a voltage clamp implementation. Preferably, the assay is performed about 24 hours to 72 hours, preferably 30 to 60 hours and more preferably
15 42 to 50 hours after introduction of the transgene. Preferred cells include cerebellar granule neurons and adult cardiac myocytes. See the examples which follow.

All documents mentioned herein are incorporated herein by reference.

The invention is further illustrated by the following non-limiting examples.

Example 1- Production of Constructs Overexpressing an HERG Potassium Current
20 Gene

The HERG gene encodes a potassium current known as I_k . The HERG gene was overexpressed in canine ventricular myocytes using viral gene transfer manipulations described above.

Example 2- Result of Overexpressing an HERG Potassium Current Gene

25 The vector constructs prepared in Example 1 were introduced into canine ventricular myocytes by somatic cell gene transfer. After 24 to 72 hours, transduced myocytes were tested by patch-clamp electrophysiology in accord with methods described above and in SoKaab et al. (1996) *Circulation Res.* 78: 262.

Figs. 2A-F compare the electrical properties of a cell infected with a reporter
30 gene (GFP) as a control (Figs. 2D-F) to those of a cell overexpressing HERG (Figs.

2A-C). Action potentials are abbreviated in the HERG-infected cell (top panels); this would be a useful therapeutic effect in patients with ventricular arrhythmias related to abnormal repolarization. The lower panels confirm that I_k has been functionally overexpressed. Thus, this cell reproduces the phenotype that would result from exposure to a selective pharmacologic agonist of HERG. Other functional tests such as numerical simulation and/or quantitation time modulation or cell or organ function could be applied to such cells, or to tissues similarly modified, in order to verify or to disprove the therapeutic utility of the agonist effect.

Example 3- Production of Constructs Expressing a Dominant Negative Kv4.2

10 Voltage-Dependent Potassium Channel Gene

Potassium channel gene products have been reported to form functional channels when four identical (or closely related) subunits come together as a tetramer; all four subunits must be functional in order for the channel complex to be functional. We have found that by deleting one or more transmembrane domains of the potassium channel, it is possible to create dominant negative mutant potassium channel proteins that block normal function of the channel complex.

There is no known pharmacological inhibitor for the transient outward current (I_{to}) formed by some potassium channel genes. Kv4.2 is an example of a voltage-dependent potassium channel gene that is expressed in mammalian heart muscle and which is believed to encode I_{to} .

There follows a description of a truncated version of Kv4.2 (Kv4.2ST). This construct was designed to multimerize with and cripple full-length products of the Kv4 gene family. The truncated construct described here includes the first transmembrane segment (S1). Thus, it was believed that Kv4.2ST would contain determinants of multimerization but would otherwise be non-functional. Such a protein would function as a dominant negative protein.

Kv4.2 and related family members were "knocked-out" by creating Kv4.2 ST. Figs. 3A-C summarizes the strategy used by presenting the Kv4.2 (SEQ ID No. 1) and Kv4.2ST (SEQ ID NO. 2) amino acid sequences and comparing those sequences by length. More specifically, Figs. 3A-C compare full-length Kv4.2 (Kv4.2FL) and

truncated Kv4.2ST proteins using a bar motif. Fig. 3B-C (SEQ ID Nos. 1,2) provide the amino acid sequences of the Kv4.2FL and Kv.2ST proteins.

Fig. 4A-B provide a schematic illustration of the full-length Kv4.2 (Fig. 4A) and Kv4.ST proteins (Fig. 4B) in a cell membrane. More particularly, Fig. 4A is a topology diagram depicting the structure of the full length Kv4.2 channel (Fig. 4A) next to the predicted structure of the Kv4.2ST protein (Fig. 4B).

The coding sequence for the rat Kv4.2 potassium channel gene was provided by M. Tamkun (Vanderbilt University). The portion of the sequence coding for the first 206 amino acids (Fig. 4A) was amplified by PCR using primers which contained unique restriction sites on the 5' and 3' ends. The product (Kv4.2ST) was cloned into pSL301 (Invitrogen, San Diego, CA) and sequenced to confirm the absence of PCR-induced mutations. The Kv4.2 ST sequence was then cloned into the adenovirus shuttle vector pE1CMV (Fig. 4B). Constructs were made in both the sense and antisense orientations with respect to the promoter, designated pE1Kv4.2ST and pE1CKv4.2AS respectively. An additional vector, pE1RKv4.2AS, contains the Rous sarcoma virus (RSV) long terminal repeat (LTR) as the promoter and was used to generate AdRKv4.2AS. The pE1Kv4.2ST vector is shown in Fig. 5. See Morgan, R.A., et al. (1992) *Nucle. Acids Res.* 20:1293; Johns, D.C., et al. (1995) *J. Clin. Invest.* 96:1152.

The 4.2ST sequence was also fused in frame to the enhanced green fluorescent protein (EGFP) sequence in the vector pEGFP-C3 (Clontech, Palo Alto, CA) to make the construct pEGFP-4.2ST. The full-length Kv4.2 sequence was subcloned into pREP4 (Invitrogen, Carlsbad, CA) and into the pEGFP-C3 backbone to make pRep4.2FL and pE-Kv4.2FL respectively. The full-length rat Kv4.3 sequence was obtained from B. Rudy (New York University Medical Center) and cloned into the expression vector pGFPIRS. This vector is a modified version of pEGFP-C3 which contains the polio virus internal ribosomal entry site (IRES), obtained from G. Ketner (Johns Hopkins School of Public Health) cloned between the EGFP sequence on the 5' side and the polycloning site on the 3' site. This vector (pGFPIrKv4.3) produces a

single transcript encoding both the EGFP protein and the Kv4.3 protein. See Morgan, R.A., et al. (1992) *Nucl. Acids Res.* 20:1293;

The human inward rectifier cDNA (Kir2.1) containing the inactivating mutation of GYG to AAA, (Tinker, A., et al. (1996) *Cell* 87:857) was provided by G. Tomaselli (Johns Hopkins University) and was fused to the EGFP sequence in pEGFP-C3 (pGFPKir2.1-AAA). The CMV- β galactosidase plasmid contains the *E. coli lacZ* gene under the control of the CMV immediate early promoter. The mitochondrial targeted GFP (mtGFP) sequence was provided by M. Rizzuto (University of Padova). The mtGFP sequence was similarly subcloned into the shuttle vector pE1CMV to make pE1CMVmtGFP. The humanized GFP was subcloned from the vector pGreenLantern (Life Technologies, Gaithersburg, MD) into pE1CMV to create pE1CMVhGFP. Mammalian expression vectors containing full-length Kv1.4 and Kv1.5 sequences were supplied by E. Levitan (University of Pittsburgh) and L. Philipson (University of Chicago), respectively. See Rizzuto, R., et al. (1995) *Curr. Biol.* 5:635; Philipson, L.H., et al. (1993) *Biochem. Biophys. Acta.* 1153:111.

Example 4 - Production of Gene Transfer Constructs

The strategy is outlined in Fig. 4B. The various adenovirus shuttle plasmids were cotransfected with pJM17, containing the full human adenovirus serotype 5 genome, (McGrory, W.J., et al. (1988) *Virology* 163:614) into HEK293 cells using Lipofectamine (Life Technologies Inc., Gaithersburg, MD). As previously described, homologous recombination between the shuttle vector and pJM17 replaces the region of the adenovirus between map units 1.0 and 9.8 with the expression cassette containing the desired cDNA. Successful recombinations were screened either by direct visualization (AdmtGFP and AdhGFP) or by Southern blot analysis of small scale infections (Ad4.2ST and AdC4.2AS and AdR4.2AS) followed by RNase protection assays of RNA made from infected cells as described below. AdCMV β -Gal contains the β -gal gene driven by the CMV promoter and was provided by G. Wilkinson (University of Wales College of Medicine, Cardiff, U.K.). The pJM17 vector is illustrated in Fig. 6.

Using standard cloning methods, the Kv4.2 ST was inserted into adenovirus to form a replication-deficient recombinant gene transfer construct, AdKv4.2ST. The recombination strategy is outlined in Fig. 7A and 7B.

5 Figs. 7A and 7B are further explained as follows. Cotransfection of the adenoviral shuttle vector pE1Kv4.2St and the bacterial plasmid pJM17 in HEK293 cells results in a recombinant adenovirus. This recombination replaces the E1 region of the adenovirus with Kv4.2ST expression cassette.

The somatic cell gene transfer protocol enables efficient infection of a variety of tissues, including the heart.

10 Example 5 - Molecular Analysis of Expressed Potassium Channel Genes

Viral DNA was isolated as previously described (Hitt, M., et al. (1994) *Cell Biology: A Laboratory Handbook* pp.479). DNA was size separated by agarose gel electrophoresis and blotted onto Nytran nylon membranes using a Turboblotter apparatus (Schleicher and Schuell, Keene, NH). Blots were pre-hybridized and
15 hybridized in Rapid-hyb buffer (Amersham Life Sciences, Arlington Heights, IL). Probes were labeled using a random primed labeling kit (Boehringer-Mannheim, Indianapolis, IN) and (α -P³²) dCTP (New England Nuclear, Boston, MA).

Total RNA was isolated from HEK293 cells using Trizol reagent (Life Technologies, Gaithersburg, MD). Ribonuclease protection assays were performed
20 using the RPAII kit (Ambion, Austin, TX). Ten micrograms of total RNA was hybridized to both sense and antisense RNA probes for the Kv4.2ST sequence. Protected fragments were separated on a 5% denaturing polyacrylamide gel.

Southern blot analysis of isolated viral DNA confirmed the presence of the Kv4.2ST sequence in the recombinant adenovirus. RNase protection analysis of total
25 RNA isolated from HEK293 cells which had been infected with either AdKv4.2ST, AdCKv4.2AS or AdRKv4.2AS revealed that only sense and antisense (respectively) RNAs were expressed from these constructs in detectable amounts (see Fig. 8).

FIG.8 is explained as follows. RNase protection assay using both sense and anti-sense probes (Lanes 1,6) hybridized to yeast RNA (lanes 2 and 7), total RNA
30 from HEK293 cells infected with AdKv4.2ST (lanes 3 and 8), or total RNA from cells

infected with AdRKv4.2AS or AdCKv4.2AS (lanes 4&9 and 5&10 respectively).

Fig. 8 shows that AdKv4.2ST expresses only the sense strand of the Kv4.2ST sequence

Example 6 - Transfection of Kv4.2 Potassium Current Genes into CHO-K1 cells

5 CHO-K1 cells (CCL-61 American Type Culture Collection, Rockville, MD) were grown in Ham's F-12 media (CellGro, Mediatech, Washington D.C.) supplemented with 10% fetal bovine serum (LifeTechnologies, Gaithersburg, MD) at 37C° in a 5% CO₂, humidified incubator. Cells were split and plated at 30-40% confluency on coverslips in six well plates 24 hours before transfection. Cells were
10 transfected with plasmid DNA (1µg/well total) using Lipofectamine (LifeTechnologies, Gaithersburg, MD). After a four-hour exposure cells were washed once with normal growth media and then incubated for one to two days in normal growth media under normal growth conditions.

 When cotransfected into CHO-K1 cells, Kv4.2ST suppresses functional
15 expression of a full-length Kv4.2 construct (Fig. 9A,B,C). These experiments were done at a 2:1 molar ratio of pE1Kv4.2ST: pRepKv4.2FL based on preliminary experiments in which the currents were found to be much more variable at molar ratios of 1:1 and 1:2. Suppression of Kv4 current is specific, as cotransfection of Kv4.2ST with a Kv1.5 expression vector (also at a 2:1 molar ratio) does not affect
20 functional expression of this channel (Fig. 9D,E, F). These results encouraged the production of the recombinant adenovirus containing this construct (AdKv4.2ST), for infection of neurons and cardiac myocytes which are notoriously difficult to transfect by conventional means.

 Figs. 9A-F are explained as follows. Wild type Kv4.2 currents expressed in
25 CHO-K1 cells co-transfected with pCMVβ-Gal (Fig. 9A) or pE1Kv4.2ST (Fig. 9B), currents were elicited at +40 mV. Summary data for peak current densities (Fig. 9C) were obtained by subtracting the prepulse inactivated current (0 mV prepulse) from the fully primed current (-90 mV prepulse). The magnitude of Kv1.5 current expressed in CHO-K1 cells is not affected by cotransfection with Kv4.2ST (Fig. 9D

vs. Fig. 9E). The mean data shown in (Fig. 9F) summarizes the results of 5+6 experiments.

Example 7 - Somatic Cell Gene Transfer of Modified Potassium Current Genes

Isolation of Cerebellar Granule Cells- Cerebellar granule cells were isolated as previously described Levi, G., et al. (1989) *Manual of the Nervous System* pp. 211. Cells were plated on dishes that had been coated with poly-L-lysine (5ug/ml for 10 minutes). If coverslips were needed they were sterilized by immersion in 95% EtOH followed by flaming in a bunsen burner. Coverslips were then placed in the dishes prior to coating with poly-lysine. Cells were cultured in Basal Eagle's medium with Earle's salts free of glutamine (Life Technologies Inc., Gaithersburg, MD) that had been supplemented with 10% heat inactivated (HI) FBS, 100 µg/ml gentamicin, 25 mM KCl, and 2mM L-glutamine . The media was removed after one day and replaced with fresh media containing 10uM cytosine arabinoside (AraC). Under these culture conditions, expression of the A-type current is stable throughout the course of the experiment Gorter, J.A., et al. (1995) *J. Neurophysiol.* 74:298.

Isolation of Ventricular myocyte cells- Adult rat ventricular myocytes were isolated by enzymatic dissociation as previously described Hilal-Dandan, R., et al. (1991) *J. Mol. Cell Cardiol.* 23:705. Cells were cultured on laminin-coated coverslips in Medium 199 with Earles salts and L-glutamine (CellGro, Mediatech, Washington D.C.) supplemented with penicillin, streptomycin, and 4% fetal bovine serum (LifeTechnologies, Gaithersburg, MD).

Cultured rat cerebellar granule cells were co-infected with a 3:1 ratio of Ad4.2ST or Adβ-Gal: AdhGFP, on day three in culture. The multiplicity of infection (m.o.i.) which gave best results was in the range of 100 to 500. The AdhGFP allowed visual inspection of the percent infected cells. Infections were carried out in a minimal volume of culture media supplemented with HI 2% FBS for two hours at 37° C . The best results were obtained by removing a cover slip of cells from the dish and placing it into a fresh dish where it was covered with 100 to 200 µl of infection media. Surface tension was sufficient to hold the infection media in place. Following the 2 hour infection time the coverslip was placed back in the original culture plate and

monitored for the appearance of GFP in granule cells (24-48 hrs.). If the cells had been co-infected with β -galactosidase they were further processed by fixing in 4% paraformaldehyde and stained for β -galactosidase activity.

Cerebellar granule neurons were chosen to test the idea that the A-type current is encoded by *Shal* family genes (Serodio, P., et al. (1996) *J. Neurophys.* 75:2174; Gorter, J.A., et al. (1995) *J. Neurophysiol.* 74:298; Zegarra-Moran, O., et al. (1994) *Exp. Brain Res.* 98:298). In addition, these cells are easily maintained in culture with minimal changes in A-type current density over time. Infection of cultures of granule neurons using low m.o.i.'s (10-50) of AdhGFP resulted primarily in infection of surrounding glial cells, as had been previously observed in other neuronal cultures Moriyoshi, K., et al. (1996) *Neuron* 16:255. Granule cells are easily distinguished from glial cells by their size and morphology. When moi's were increased to 100-500, infection of neuronal cells could be observed at 24 to 48 hours. To positively identify infected neurons for electrophysiologic study, AdhGFP was included in all infections and the duration of expression was calculated from the first appearance of GFP-positive neurons.

Rat ventricular myocytes were similarly infected except that the m.o.i.'s used were in the range of 5 to 20 and it was not necessary to infect a single coverslip at a time as there were no surrounding cells as with neuronal culture. AdmtGFP was used to monitor infection efficiency for myocytes rather than AdhGFP.

Rat ventricular myocytes were also chosen due to the established presence of both Kv4.2 and Kv4.3 mRNA in the heart, and the relative ease with which these cells could be maintained for several days in primary culture Dixon, J.E., et al. (1994) *Circ. Res.* 75:252; Dixon, J.E., et al. (1996) *Circ. Res.* 79:659.

Example 7- Electrophysiology of Constructs Expressing the Kv4.2 ST Potassium Channel Gene

The following experiments verify that Kv4.2 ST works as designed in transient cotransfection experiments with functional Kv4.2.

Experiments were performed at 21-23°C. Whole-cell currents were recorded with Axopatch 200A amplifier (Axon Instruments, Foster City, CA) sampled at 10

kHz and filtered at 1-5 kHz. The pipette solution contained (in mM) 140 KCl, 1 MgCl₂, 5 EGTA, 4 MgATP, 10 HEPES (pH 7.2). The use of intracellular EGTA suppressed any calcium-dependent component of the transient outward current Zygmunt, A.C., et al. (1991) *Circulation Research* 68:424, which might have been present in the rat ventricular myocytes; the remaining I_{to} thus consists entirely of the calcium-independent transient outward potassium current (also called I_{to1}). The bath solution contained (in mM) 140 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES (pH7.4). For comparison between groups, peak currents are normalized for cell size based on the capacitance of the cell and expressed as current density (pA/pF).

10 To reduce experimental variability in cotransfections, cells were routinely studied between 42 and 50 hours after the introduction of the transgene. Viral infections of primary cells were also studied at this time, for consistency with the cotransfection and for the following practical reasons. In the case of adult cardiac myocytes, variable changes in ion channel expression with increasing time in culture were too great to allow statistical evaluation beyond 2-3 days. The cerebellar granule neurons began to exhibit cytopathic effects at time points past 72 hours of infection in both control and test infections.

Figs. 10A-B show current density in controls (10B) and in Kv4.2 ST-cotransfected CHO cells (10A). Cotransfection of Kv4.2 ST prevented the expression of functional channels.

Using the somatic cell gene transfer protocol disclosed above, heart cells were infected (canine ventricular myocytes) with AdKv4.2ST. It was found that the transient outward current was eliminated (Figs. 11A-B). The effect was selective for the transient outward current. The Kv4 gene family has been proposed to underlie the transient outward current, although this had never been demonstrated using a dominant negative approach. Thus, this verifies that truncated channel genes can be used to knock out endogenous currents in mammalian heart cells. The approach can thus be used to predict the functional consequences of a drug designed to suppress the transient outward current.

Figure 12A shows representative A-type currents elicited in an Ad β -Gal-infected control cell upon depolarization to +40 mV following prepulses to two different potentials (-90mV, -40mV, 500 mS). Infection with AdKv4.2ST suppresses the A-type current in the cerebellar granule neurons, without affecting the maintained component (Fig. 12B). The pooled data from 8 control and 8 test cells (Fig.12C) confirm that the reduction in current density by AdKv4.2ST is significant ($p<0.001$). The virally-mediated suppression of native A-type current in neurons is not complete at 42 to 50 hours, unlike the suppression of the expressed Kv4.2 current (Fig.9A-F). The remaining transient currents decay at the same rate as the control currents, suggesting that the residual current is comprised of channels that are functionally identical to those which were knocked out. Thus, the lack of complete knockout likely reflects competition between the time course of expression of the Kv4.2ST gene product and the turnover rate of the functional channel subunits. Alternatively, it is possible that other, non-Kv4 family genes encode a minor fraction of the A-type current.

Figs. 12A-C are explained as follows. Cerebellar granule neurons infected with AdCMV β -Gal (Fig. 12A) exhibit large transient and maintained outward currents upon depolarization to +40 mV. The transient component of current can be inactivated with a pre-pulse to -40 mV. AdKv4.2ST infection (Fig. 12B) results in a marked suppression of the transient component without affecting the maintained component of outward current. Results of 8 experiments are summarized in Fig. 12C. Figs. 12A-C show that AdKv4.2ST suppresses the A-type current of cerebellar granule neurons.

Fig. 13A-C show the fully primed and pre-pulse inactivated (-90mV, -40mV) transient outward currents elicited by test pulses to +40mV in a myocyte infected with Ad β gal (Fig. 13A) and in another infected with AdKv4.2ST (Fig. 13B). The pooled data in Fig. 13C (N=6 in each group) confirm the significant suppression of native rat cardiac I_{to} by infection with AdKv4.2ST. As was the case with the cerebellar granule cells, the suppression of current was substantial but not complete at

42 to 50 hours. Nevertheless, the results indicate that Kv4 genes constitute the major contributors to I_{to} in heart cells and to A-type currents in cerebellar granule cells.

Figs. 13A-C are described as follows. Ca^{2+} -independent transient outward currents at +40 mV recorded in AdCMV β -Gal infected myocytes are compared to currents in AdKv4.2ST infected myocytes (Fig. 13B). Peak I_{to} current was measured as the difference between pre-pulse inactivated (0 mV) and fully primed currents. A marked suppression in the peak current density is indicated in the summary data (Fig. 13C). Figs. 13A-C illustrate that AdKv4.2ST suppresses I_{to1} in adult rat ventricular myocytes.

10 Example 8 - Analysis of Constructs Encoding Fused Kv4.2-GFP Fusion Proteins

Electrophysiology- To probe the mechanism of action of Kv4.2ST, fusion constructs were generated with enhanced green fluorescent protein (EGFP) so that the expressed truncated protein could be localized within living cells with confocal imaging. We first confirmed that the fusion protein GFP-Kv4.2ST acted similarly to Kv4.2ST (Figs. 14A-I).

Figs. 14A and 14B show currents recorded in a CHO-K1 cell co-transfected with pRCMVKv1.4 and pCMV β -Gal (Fig. 14A) or pGFPKv4.2ST (Fig. 14B) . As was the case for Kv4.2ST and Kv1.5, there is no suppression of Kv1.4 by GFP-Kv4.2 (Fig. 14C). Nevertheless, the fusion protein could suppress *Shal* family currents (either Kv4.2, Fig. 14 D-F, or Kv4.3, Figs. 14 G-I). The summary data in Figs. 14 F and I show comparable levels of suppression for Kv4.2 and Kv4.3 ($P \leq 0.005$).

Kir2.1-AAA is an unrelated inwardly rectifying potassium channel with a pore mutation designed to suppress Kir2.1 channels Tinker, A., et al. (1996) *Cell* 87:857, and was added as a control in these experiments to ensure that suppression of Kv4.2 current was not simply attributable to co-expression with another membrane protein. This control is particularly apt because GFPKir2.1-AAA does suppress the functional expression of inwardly rectifying currents encoded by wild-type Kir2.1, Schreier, K., et al. (1997) *Circulation* 96:1-422.

Figs 14A-I are explained as follows. Currents recorded from CHO-K1 cells co-transfected with pRC-CMVKv1.4 and pCMV β -Gal (Fig 14 A) or pGFP-Kv4.2ST

(Fig. 14B) are not significantly different (n=5)(Fig. 14C). Wild type Kv4.2 currents from CHO-K1 cells co-transfected with a dominant negative construct for Kir2.1 (pGFPKir2.1-AAA) (Fig. 14D) are compared to currents in cells co-transfected with pGFP-Kv4.2ST (Fig. 14E). The fusion construct containing Kv4.2ST causes a large reduction in average current density (n=3) (Fig. 14F). This effect is mimicked when a Kv4.3 expression vector (pGFP_{IR}Kv4.3) is used in place of pE-Kv4.2FL (Figs. 14G, H). The reduction in Kv4.3 current achieved by GFP-Kv4.2ST in four experiments is significant (Fig. 14I). Figs 14A-I show that a modified Kv4.2ST (GFP-Kv4.2ST) also specifically suppresses *Shal* family currents.

Confocal Microscopy- CHO-K1 cells (CCL-61 American Type Culture Collection, Rockville, MD) plated on glass coverslips were transfected with pGFP-Kv4.2ST or with pGFP-Kv4.2ST and pE-Kv4.2FL using Lipofectamine. After 36 hours cells were washed with PBS and placed upside down on a microscope slide over a drop of PBS. The edges of the coverslip were sealed with rubber cement to prevent drying. Images were taken on a laser scanning confocal microscope (PCM 2000, Nikon Inc., Melville, NY, EX 488, EM 505-530) with a 60X objective lens (NA 1.2).

Confocal imaging of CHO-K1 cells transfected with pGFP-Kv4.2ST (Fig. 15A) or cotransfected with pGFP-Kv4.2ST and pE-Kv4.2FL (Fig.15B) reveals that the fusion construct is richly concentrated in the perinuclear region of the cells. In families of Z-plane images fluorescence intensity was also detected on the surface of the cells. These findings suggest that, at least some of the suppression of functional current may be due to premature degradation of heteromeric channel complexes, and/or to effects on the processing of the mature protein prior to externalization.

However, this apparently abnormal localization of a membrane protein does not seem to be restricted to the truncated version of this protein, as fusion constructs containing full-length channels also have similar localization patterns despite the fact that robust membrane currents can be readily detected in such cells (Marshall, J. et al. (1995) *Neuron* 14:211). Therefore, it is not possible at this time to state unequivocally whether the suppression of current occurs as a result of premature degradation, as the

result of the formation of non-functional tetramers in the surface membrane, or as a combination of the two effects.

Figs. 15A and B are explained as follows. A confocal image of pGFP-Kv4.2ST transfected into CHO-K1 cells (Fig. 15A) shows concentration of the fusion protein in the intracellular membranes. This distribution is similar in a cell that was cotransfected with pGFP-Kv4.2ST and pE-Kv4.2FL (Fig. 15B). Figs. 15A and 15B show that GFP-Kv4.2ST is distributed in various cell membranes.

The examples shown above demonstrate use of a dominant negative Kv4.2 ion channel construct that specifically suppresses the transient outward current of rat ventricular myocytes as well as the A-type current of cerebellar granule neurons.

These two cell types have previously been shown by other methods to express Kv4 family genes. See Dixon, J.E., et al. (1994) *Circ. Res.* 75:252; Barry, D.M., et al. (1995) *Circ. Res.* 77:361; Dixon, J.E., et al. (1996) *Circ. Res.* 79:659; Serodio, P., et al. (1996) *J. Neurophys.* 75:2174; Maletic-Savatic, M., et al. (1995) *J. Neurosci* 15:3840; Fiset, C., et al. (1997) *Journal of Physiology* 500:51.

It is shown that Kv4.2ST specifically suppresses members of the Shal family. It is believed that this is the first experimental demonstration that *Shal* family members can form heteromultimers with each other. This strategy provides a unique way of determining the molecular identity of macroscopic ionic currents in native cells and may provide a useful tool in understanding the exact role these currents play in cellular physiology. The use of the adenovirus vector also allows the potential use of this strategy *in-vivo* as well as *in-vitro*. While the introduction of dominant negative constructs may also be achieved by transgenic approaches, developmental adaptation or possible lethal effects may complicate the interpretation of such experiments.

Some of the examples disclosed above use statistical analysis to present data. Statistical analyses were achieved by pooling relevant data as the mean \pm the standard error of the mean (s.e.). Comparisons of means between groups were performed using one way ANOVA. *P* values less than 0.05 were deemed significant.

The following abbreviations were used in the text: I_{to} and I_A , transient outward current; CMV, cytomegalovirus; RSV, rous sarcoma virus; LTR, long terminal repeat; IRES, internal ribosome entry site; m.o.i., multiplicity of infection; EGFP, enhanced green fluorescent protein; AraC, cytosine arabinoside, β -gal, *Escherichia coli lacZ* gene; pF, picofarad.

The invention has been described with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. A method for predicting the effect of a drug candidate compound, comprising:
 - a) modulating, by somatic gene transfer, expression of a selected protein in selected cells; and
 - b) analyzing the result of expression of the protein to thereby predict the effect of the drug candidate compound.
2. The method of claim 1 wherein the result of the protein expression is analyzed to identify a molecular target of the drug candidate compound.
3. The method of claim 1 wherein the result of the protein expression simulates the predicted pharmacological effect of the drug candidate compound.
4. The method of claim 1 wherein the result of the protein expression alters function of the cells.
5. The method of claim 1 wherein a function of the cells is suppressed by the protein expression.
6. The method of claim 1 wherein a function of the cells is induced by the protein expression.
7. A method for detecting a potential drug target protein, comprising modulating, by somatic gene transfer, expression of the target protein in selected cells, and analyzing the result of expression of the target protein.
8. The method of claim 7 wherein expression of the target protein is increased following the somatic gene transfer.
9. The method of claim 8, wherein the increase in expression is achieved by operably linking a gene encoding the target protein to an inducible or viral promoter.
10. The method of claim 7 wherein expression of the target protein is inhibited following the somatic gene transfer.
11. The method of claim 10 wherein expression of the target protein is inhibited by transfer of a gene truncated relative to a corresponding native gene.

12. The method of claim 11, wherein the truncation is a contiguous or non-contiguous deletion of the transferred gene.

13. The method of claim 10, wherein expression of the target protein is inhibited by transfer of a gene encoding one or more amino acid substitutions relative to a corresponding native protein.

14. The method of claim 7, wherein the target protein is capable of specifically forming a binding complex with at least one other protein.

15. The method of claim 14, wherein expression of the target protein is sufficient to produce a dominant negative mutation that reduces or blocks function of the binding complex.

16. The method of claim 7, wherein the potential drug target protein is an ion channel protein, the selected cells are capable of producing an ion current, and the analysis comprises measuring the ion current produced by the selected cells.

17. A method of detecting an ion channel capable of serving as a drug target protein, the method comprising:

- a) providing a population of somatic cells capable of producing an ion current from a recombinant nucleic acid segment encoding an ion channel protein;
- b) modifying the nucleic acid segment sufficient to alter the ion current produced by the encoded ion channel protein;
- c) transferring the modified nucleic acid segment into the somatic cells under somatic cell gene transfer conditions which allow expression of the encoded ion channel and production of an altered ion current;
- d) detecting the altered ion current; and
- e) correlating the altered ion current to the capacity of the ion channel protein to serve as the drug target protein.

18. The method of claim 17 wherein expression of the ion channel protein is increased following the somatic gene transfer.

19. The method of claim 17 wherein expression of the target protein is inhibited following the somatic gene transfer.

20. The method of claim 19 wherein expression of the target protein is inhibited by transfer of a gene truncated relative to a corresponding native gene.

21. The method of claim 20, wherein the truncation is a contiguous or non-contiguous deletion of the transferred gene.

22. The method of claim 19, wherein expression of the target protein is inhibited by transfer of a gene encoding a modified protein comprising one or more amino acid substitutions relative to a corresponding native protein.

23. The method of claim 19, wherein the target protein is capable of forming a binding complex with at least one other protein.

24. The method of claim 23, wherein expression of the target protein is sufficient to produce a dominant negative protein that reduces or blocks function of the binding complex.

25. A method of reproducing a cardiac arrhythmia phenotype in a population of cultured cells, the method comprising:

a) providing a population of cultured somatic cells capable of producing an ion current from a recombinant nucleic acid segment encoding an ion channel protein;

b) modifying the nucleic acid segment sufficient to alter the ion current produced by the encoded ion channel protein;

c) transferring the modified nucleic acid segment into the cells under conditions which allow expression of the encoded ion channel and production of the altered ion current;

d) detecting the altered ion current; and

e) correlating the altered ion current to the capacity of the ion channel to serve as the therapeutic target.

26. A method of reproducing a cardiac arrhythmia phenotype in a population of cultured cells, the method comprising:

a) providing a population of cultured somatic cells capable of producing an ion current from a recombinant nucleic acid segment encoding an ion channel protein;

- b) modifying the nucleic acid segment sufficient to alter the ion current produced by the encoded ion channel protein;
- c) transferring the modified nucleic acid segment into the cells under conditions which allow expression of the encoded ion channel and production of the altered ion current; and
- d) detecting the altered ion current sufficient to reproduce the mammalian cardiac arrhythmia in the cultured cells.

27. The method of claim 25 or 26 wherein the modification of the nucleic acid segment overexpresses the ion channel protein in the population of somatic cells.

28. The method of claim 25 or 26 wherein the modification of the nucleic acid segment produces a dominant negative ion channel proteins.

29. A method of mimicking one or more effects of a drug candidate compound in an identified somatic cell, tissue or organ of interest, the method comprising:

- a) modulating, by somatic gene transfer, expression of a selected protein in selected cells; and
- b) analyzing the result of expression of the protein to thereby predict the effect of the drug candidate compound.

30. The method of claim 29, wherein the drug candidate compound would increase or suppress activity of the protein in the cell, tissue or organ.

31. The method of claim 30, wherein the method further comprises use of a standard drug discovery strategy and use of the protein identified by the method.

ABSTRACT

The present invention relates to somatic cell gene transfer methods for mimicking one or more effects of a drug candidate compound. In one aspect, the methods mimic the effect of a drug candidate compound with potential to potentiate or suppress activity of a selected target molecule. In another aspect, the methods provide means of identifying a molecular target for the drug candidate compound. The present methods have a variety of uses including providing identified molecular targets for use in drug screens.

FIGURE 1-A

PROTEIN	DESCRIPTION	REFERENCE ¹	mRNA/gene Sequence ²
Voltage-Gated Ion Channel	K ⁺ channel beta 1a subunit	Hs.45090	U33428 L39833 L47665
Sodium Channel	Voltage dependant sodium channel		
Calcium Channel	Dihydropyridine-Sensitive L-Type, Calcium Channel Beta-1-B1 Subunit	Hs.635	L0611 M92392 M76560
	Dihydropyridine-sensitive L-type calcium channel alpha-1 subunit (CACNL1A3),	Hs.1294	L33798 U30707
	Neuronal DHP-sensitive, voltage-dependent, calcium channel alpha-2b subunit	Hs.1295	M76559
	Dihydropyridine-sensitive l-type, skeletal muscle calcium channel gamma subunit	Hs.1296	L07738 Z19603
	Neuronal DHP-sensitive, voltage-dependent, calcium channel alpha-1D subunit	Hs.23838	M76558 M83556 D43747
	Putative calcium influx channel (htrp3)	H.24852	U47050 Y13758
	Voltage-dependent calcium channel alpha-1 E-3	Hs.65441	L29385 L29384 L27745
	N-type calcium channel alpha- subunit	Hs.69949	M94172 M94173
	Voltage-dependent L-type Ca channel alpha 1 subunit	Hs.89925	L29536 L29534 M92269

¹ Genbank reference designation for protein. Proteins with no designation are referenced in this text.

² Genbank mRNA or gene sequence reference designation.

FIGURE 1-B

PROTEIN	DESCRIPTION	REFERENCE	mRNA/gene Sequence
Ligand-Gated Channel	Gamma-amino butyric acid (GABA) receptor		
	Nicotinic Acetylcholine Receptor		
G-Coupled Receptor	P2Y6 receptor	Hs.16362	AF007891 U52464
	Chemokine (C-C) receptor 7	Hs.784	L08177
	Human RPE-retinal G protein-coupled receptor	Hs.1544	U14910
	Inwardly rectifying potassium channel Kir3.2	Hs.11173	U52153 D87327 U24660
	G protein-coupled receptor kinase GRK4	Hs.32859	L03718 U33054
	G protein-coupled inwardly rectifying potassium channel Kir3.4	Hs.37168	U52154
	G protein-activated inwardly rectifying potassium channel HGIRK1/Kir3.1	Hs.37169	U50964
Receptor-Gated Channel	Fc fragment of IgE, high affinity I, receptor for; beta polypeptide	Hs.30	M89796
	Interleukin 2 receptor gamma chain	Hs.84	L19546
	Cholecystokinin A receptor	Hs.129	L13605 L19315
	Peripheral-type benzodiazepine receptor	Hs.202	M36035
	Cholecystokinin B receptor	Hs.203	L07746 L10822
	Glucagon receptor	Hs.208	L20316
	Serine/threonine-protein kinase receptor R4 precursor	Hs.220	L11695
	Formyl peptide receptor-like 1	Hs.251	M84562 M88107
	Adenosine receptor A3	Hs.258	L20463 L22607

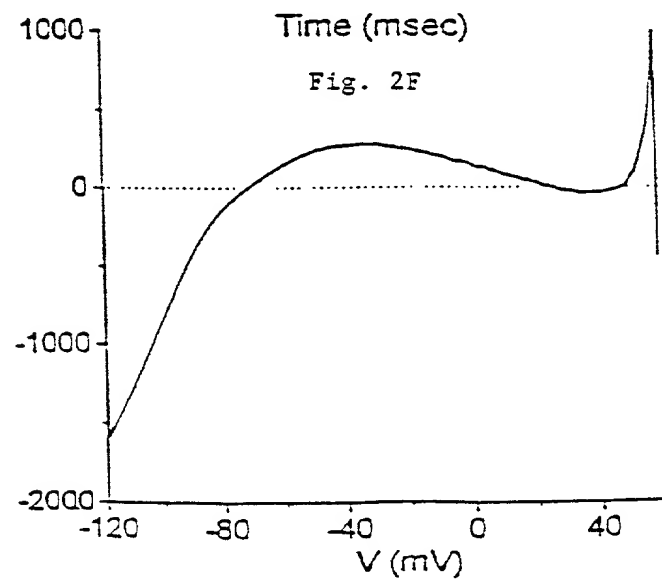
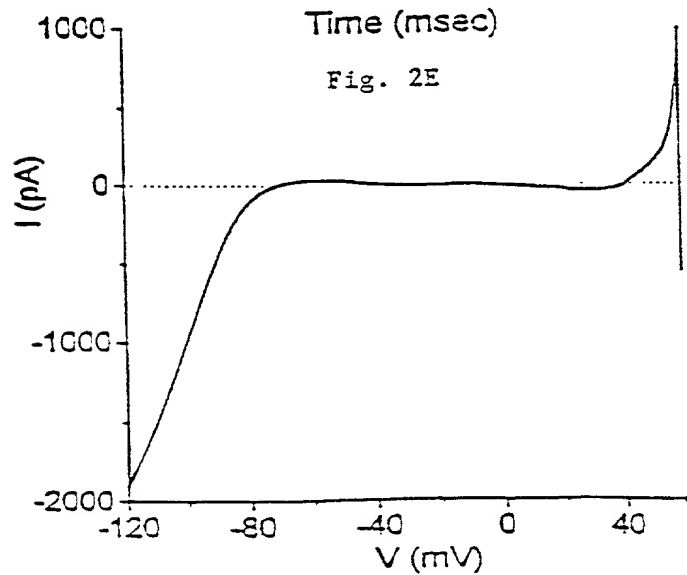
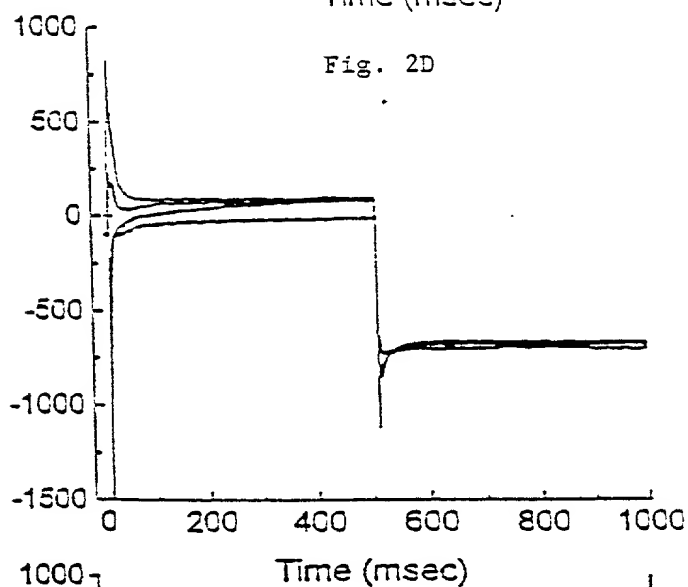
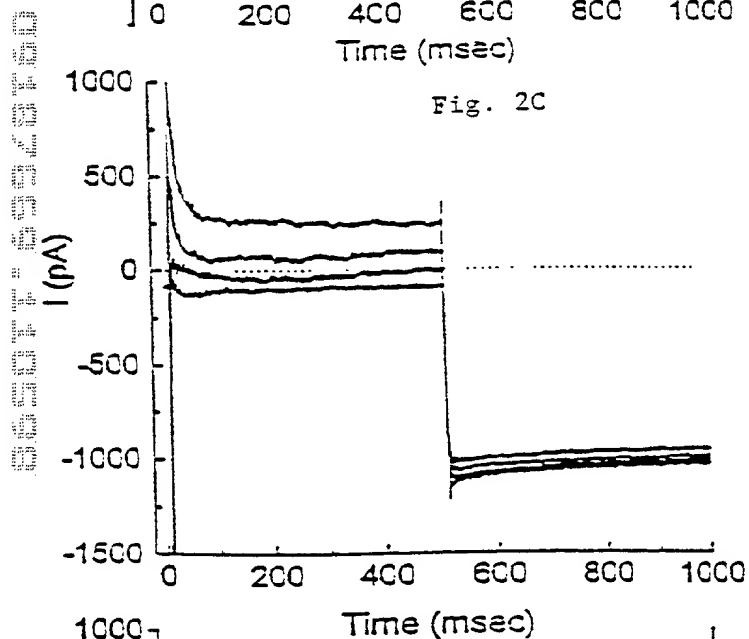
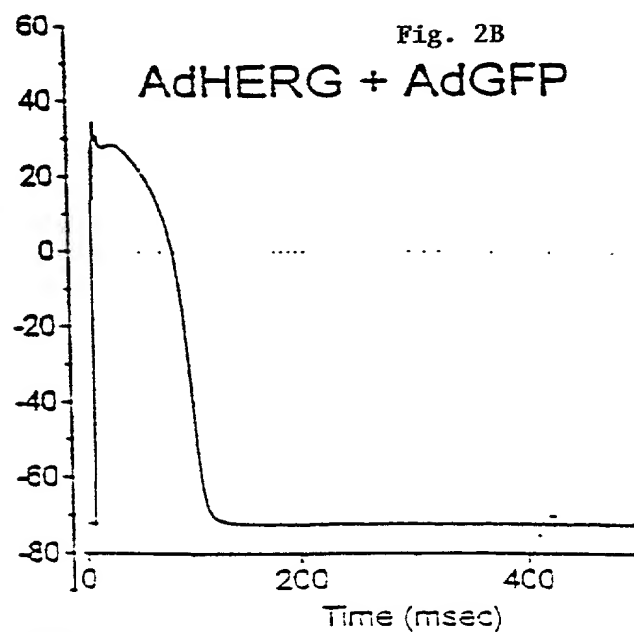
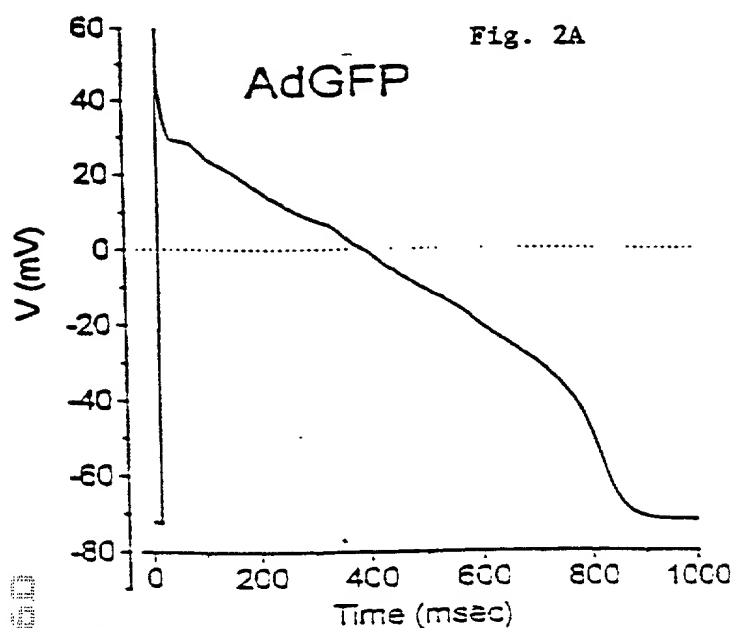
FIGURE 1-C

PROTEIN	DESCRIPTION	REFERENCE	mRNA/gene Sequence
Growth Factor Receptor	Pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	Hs.44	M57399
	Hepatocyte growth factor activator precursor	Hs.104	D14012
	Fibroblast growth factor 9 (glia-activating factor)	Hs.111	D14838
	Insulin-like growth factor binding protein 2	Hs.162	M35410
	Human growth factor receptor tyrosine kinase (STK-1)	Hs.385	U02687
Kinase	Proto-oncogene c-cot (protein-serine/threonine kinase)	Hs.248	D14497
	Receptor protein-tyrosine kinase sky	Hs.301	U18934
			D17517
	Calcium/calmodulin-dependent protein kinase IV	Hs.348	D30742
			L24959
Transferase	Creatine kinase B	Hs.669	M16451
			L47647
	V-raf murine sarcoma viral oncogene homolog B1	Hs.622	M95712
	Glucosaminyl (N-acetyl) transferase 1, core 2	Hs.781	M97347
			L41415
Isomerase	Glutathione S-transferase, microsomal	Hs.790	J03746
	UDP glucosyltransferase 8 (UDP-galactose ceramide galactosyltransferase)	Hs.57700	U30930
			U62899
	Peptidylprolyl isomerase B (cyclophilin B)	Hs.699	
	3-Beta hydroxy-5-ene steroid dehydrogenase type II	Hs.825	M67466
Protease			N77144
	Glucose phosphate isomerase	Hs.944	K03515
	Hydroxy-delta-5-steroid dehydrogenase, 3-beta- and steroid delta-isomerase 1	Hs.38586	M27137
	26S protease regulatory subunit 4	Hs.548	M38180
	Hepsin	Hs.823	L02426
Dehydrogenase	Granzyme B precursor	Hs.1051	M18930
	Glycine cleavage system protein P (glycine decarboxylase)	Hs.27	M17016
	17 beta hydroxysteroid dehydrogenase, type 2	Hs.181	M64590
			L11708

FIGURE 1-D

PROTEIN	DESCRIPTION	REFERENCE	mRNA/gene Sequence
Dehydrogenase	Xanthine dehydrogenase	Hs.250	D11456
	Alcohol dehydrogenase 7 sigma subunit (class IV)	Hs.389	U09623 U07821
	Succinate dehydrogenase 2, flavoprotein (Fp) subunit	Hs.469	D30648 L21936
Synthetase	Long chain fatty acid acyl-coA ligase	Hs.34	L09229
	Folypolyglutamate synthetase	Hs.754	M98045
	Glutamate-cysteine ligase (gamma-glutamylcysteine synthetase), catalytic	Hs.1673	M90656
	Hydroxymethylbilane synthase	Hs.82609	M95623
Deaminase	Dipeptidylpeptidase IV (CD26, adenosine deaminase complexing protein 2)	Hs.44926	M80536
	Deoxycytidylate deaminase	Hs.76894	L12136
	AMP deaminase 2	Hs.82927	U16270
	Adenosine monophosphate deaminase (isoform E)	Hs.83918	M84721
Oncogene	V-crk avian sarcoma virus CT10 oncogene homolog	Hs.16	D10656
	Thyroid hormone receptor, alpha (avian erythroblastic leukemia viral (v-erb-a) oncogene homolog)	Hs.724	M24899
	Friend leukemia virus integration 1	Hs.736	M98833
	RAP1A, member of RAS oncogene family	Hs.865	M22995
	Thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)	Hs.1166	L36051
	Fibroblast growth factor 4 (heparin secretory transforming protein 1, Kaposi sarcoma oncogene)	Hs.1755	J02986 M17446
	V-erb-a avian erythroblastic leukemia viral oncogene homolog-like 4	Hs.1939	L07868
Fos	P55-c-fos proto-oncogene protein	Hs.25647	V01512
Jun	C-jun proto oncogene (JUN)	Hs.78465	J04111
	Jun B proto-oncogene	Hs.89792	M29039

Canine LV myocytes infected with dGFP or AdHERG



Alignment

KV4, 2 TL	60
KV4, 2 ST	60

KV4.2 FL	HYDDTLGSSERDFEYHPETQOYFEDIDPDIFRUIILHLYLGLKLYYIHLHFEHDEEIN	120
KV4.2 ST	HYDDTLGSSERDFEYHPETQOYFEDIDPDIFRUIILHLYLGLKLYYIHLHFEHDEEIN	120

KV4, 2 1L
KV4, 2 5T

F₆G₆LPEIIGDCGYEEYKDKNNDEINRUODDAIDDITIGRSLIFPATTIKQIVYIKNENPTIL
FEGALPEIIGDCGYEEYKDKNNDEINRUODDAIDDITIGRSLIFPATTIKQIVYIKNENPTIL
FEGALPEIIGDCGYEEYKDKNNDEINRUODDAIDDITIGRSLIFPATTIKQIVYIKNENPTIL

100
100

[illegible]

	300
KV4.2 FI	300
KV4.2 ST	214

KV4.2 Fl. 366
 KV4.2 St 214

KV4.2 FL	fwtcivntctlgysdmvpkciagki fgsjcslsqvlvialpvpv i vnsf s r l y h q n q r a d	420
KV4.2 ST	-----	21

KV4.2 FL	krraqkariiraaksgsanaymqskrngllsnclyssedapafvksksgsfclqhh	408
KV4.2 ST	-----	21

KV4.2 FL	11hciektunhefvderyfcescmevalvnrpshps}ssqyvlalcscrhkktrf	54
KV4.2 ST	-----	21

KV4.2 FL	pnauvsghrgsvqelstlqircvertplnsrslnakneecvklnceqpyvtaltlsl	600
KV4.2 ST	-----	214

KV4.2 FL	plppvtlpegdtrpespsgnlvrsal	630
KV4.2 ST	-----	214

A

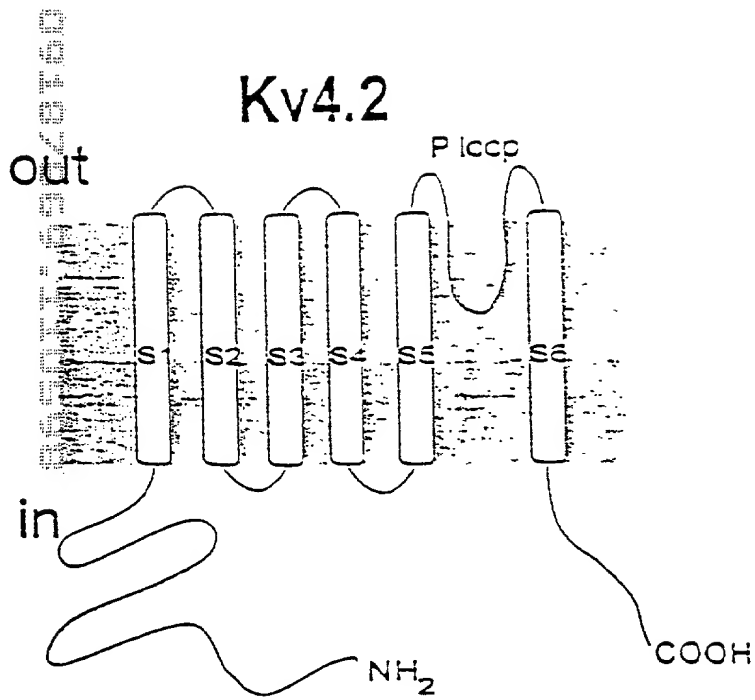


Fig. 4A

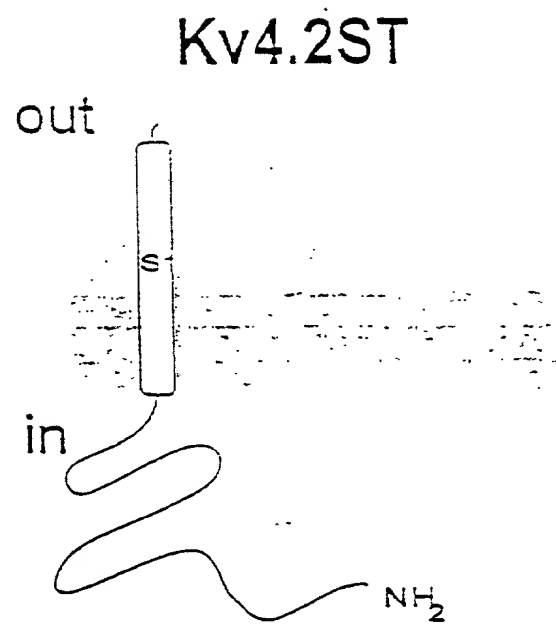


Fig. 4B

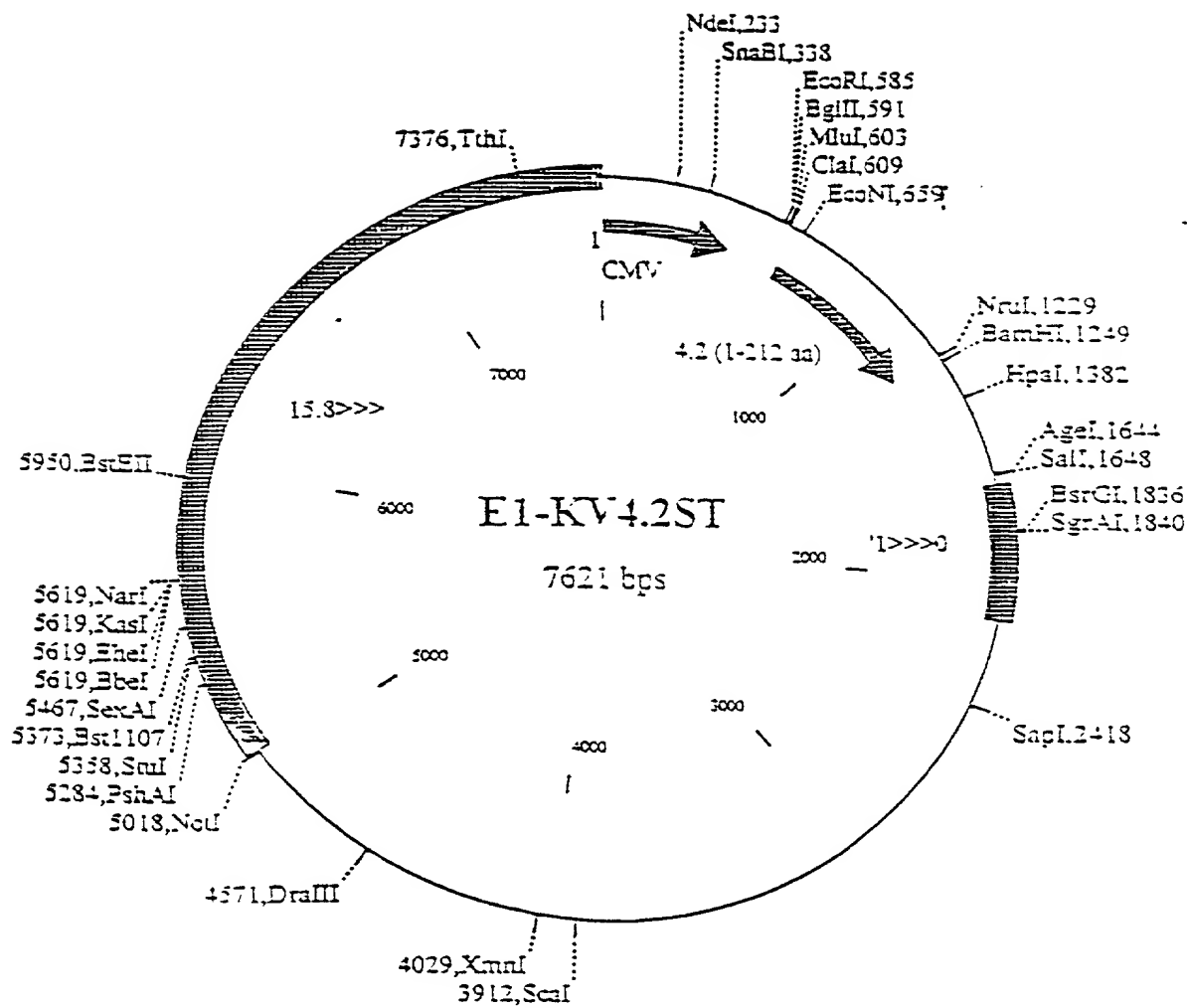


Fig. 5

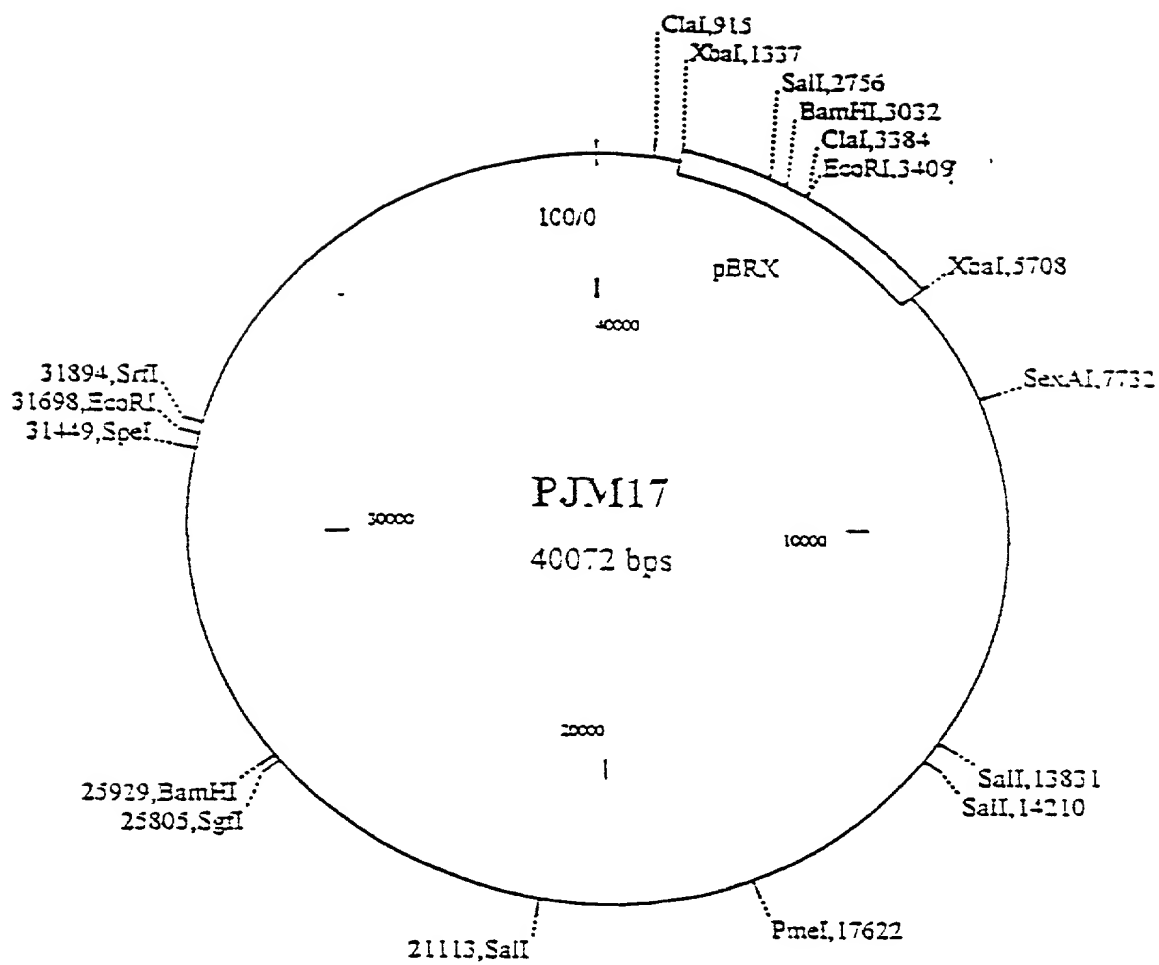


Fig. 6

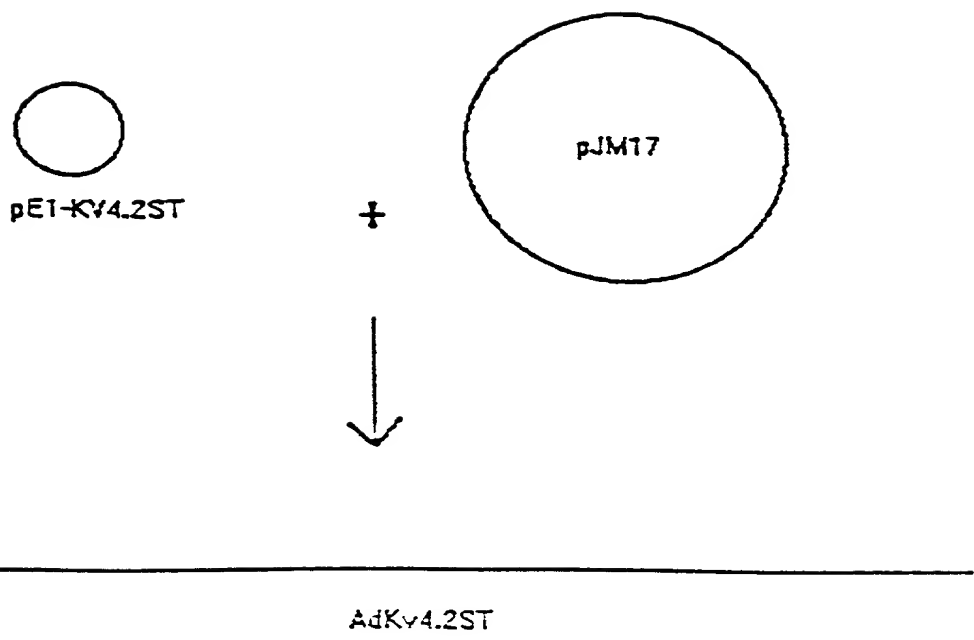
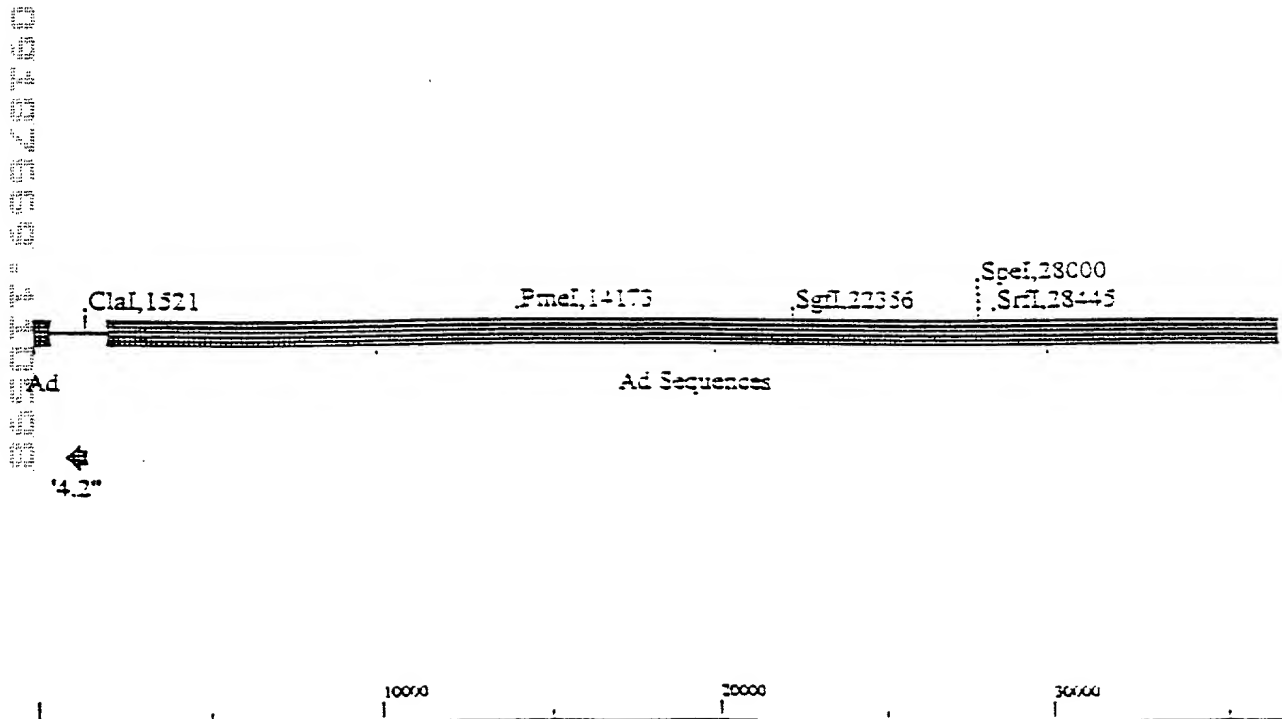


Fig. 7A



Ad42ST (36623 bps)

Fig. 7B

1 2 3 4 5 6 7 8 9 10

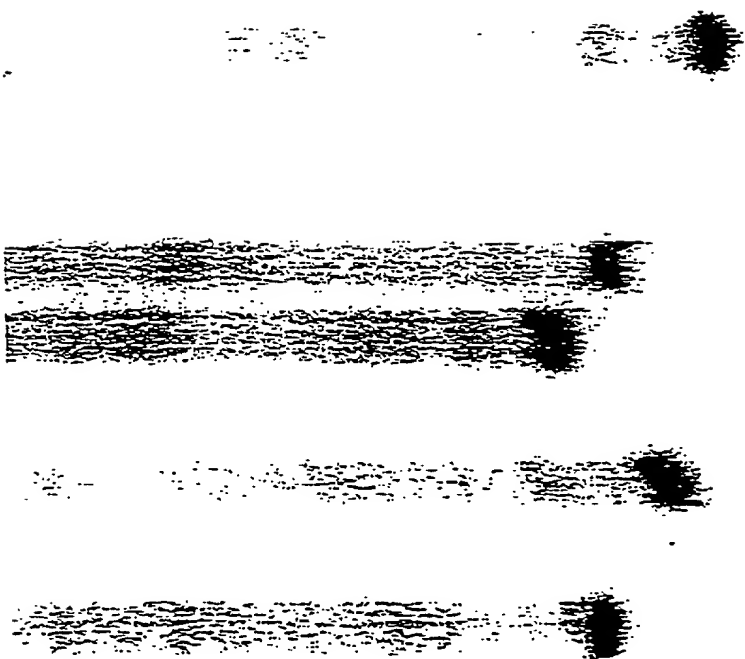


Fig. 8

09487669 440393

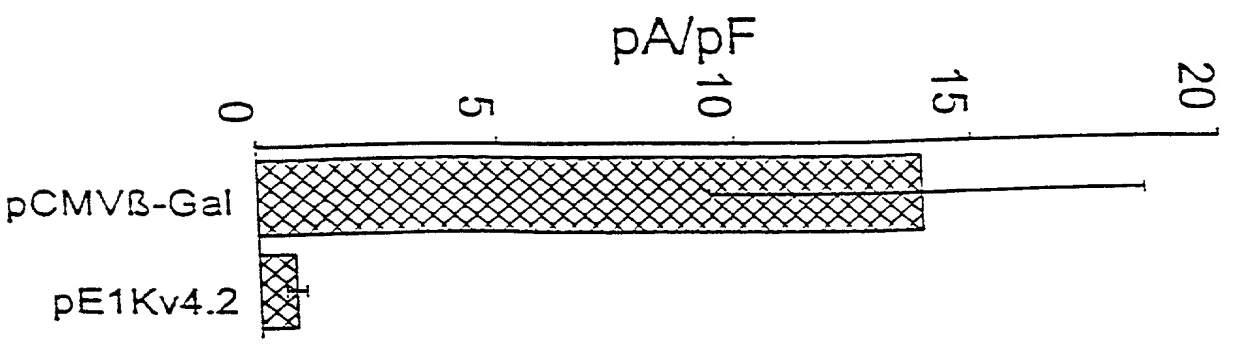
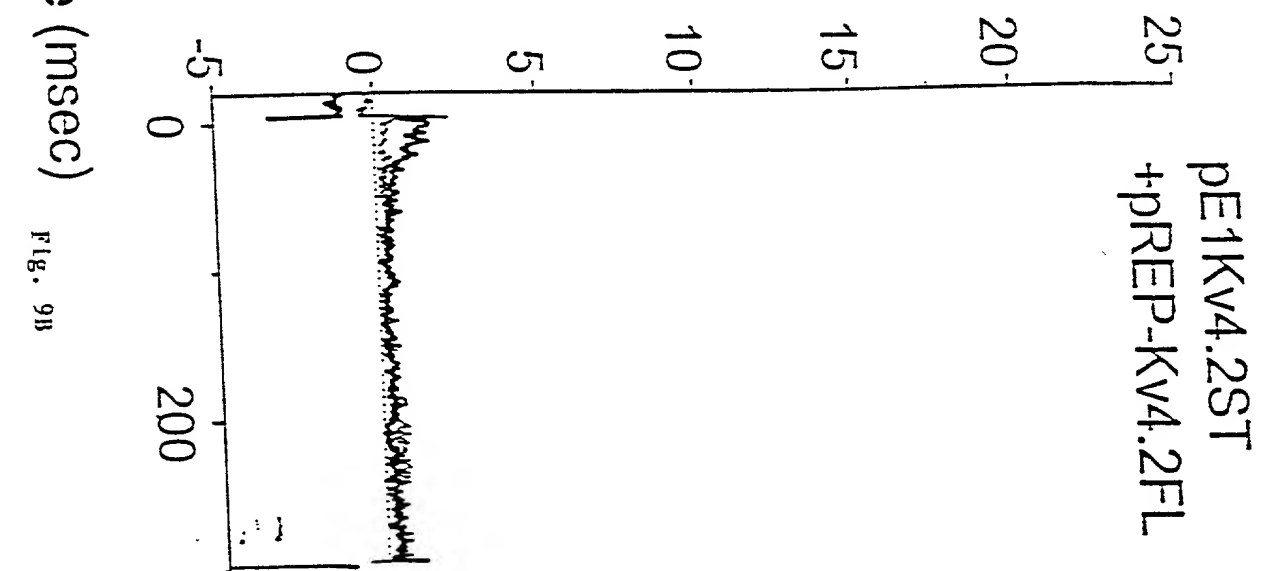
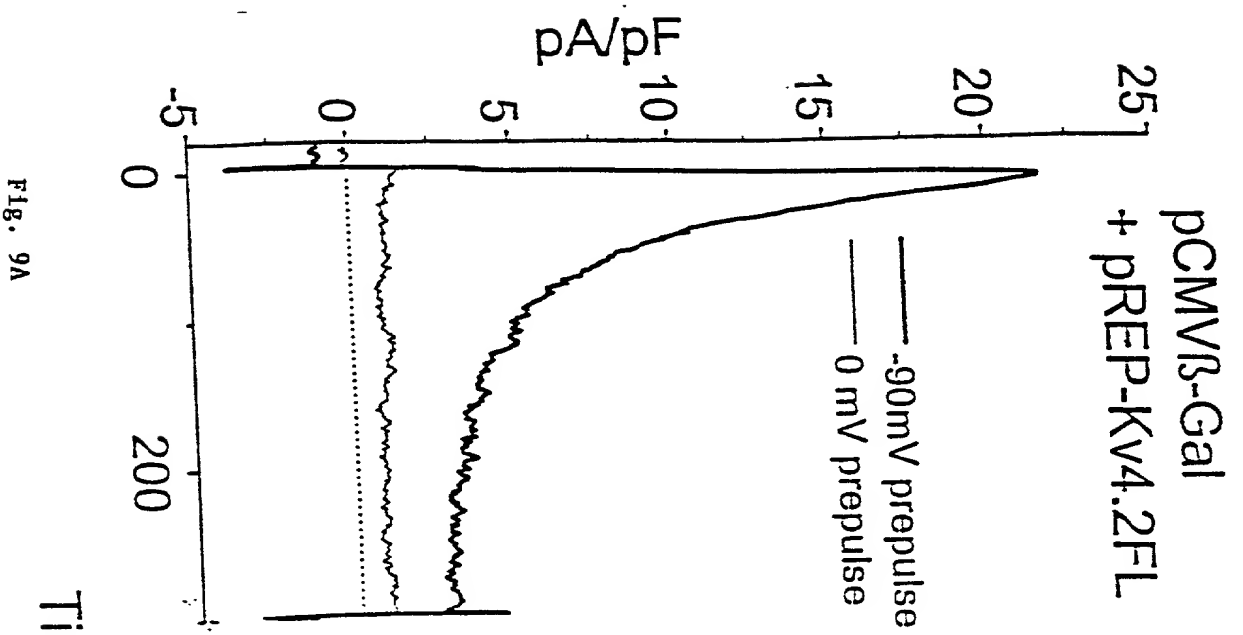


Fig. 9A

Time (msec)

Fig. 9B

Fig. 9C

09487659 440599

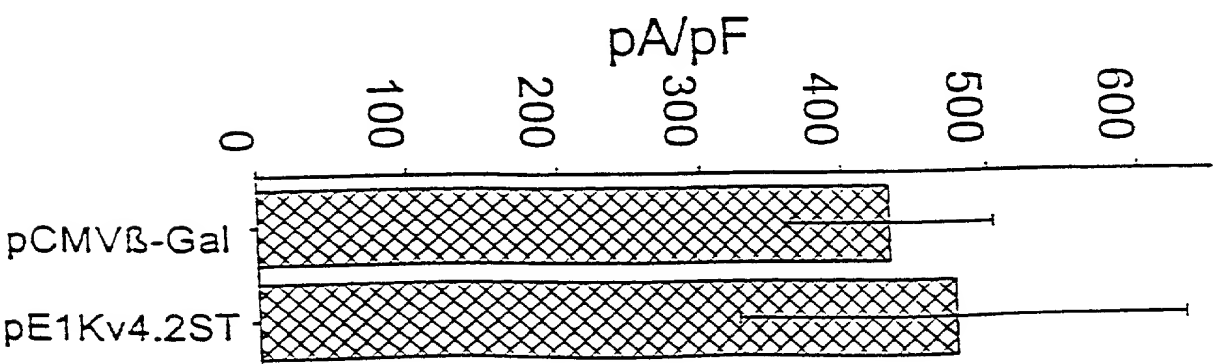
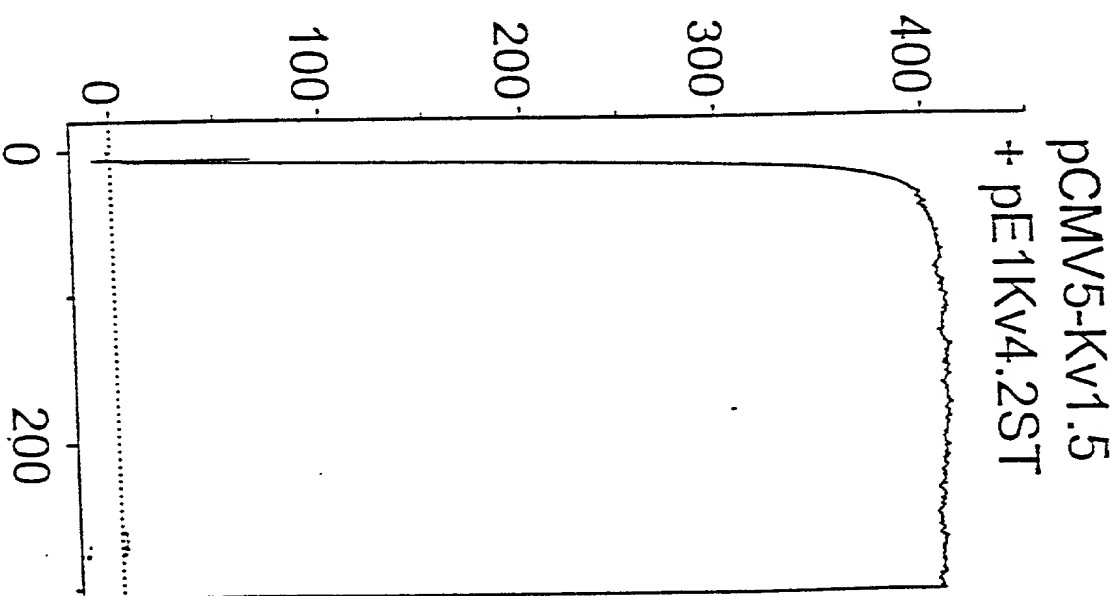
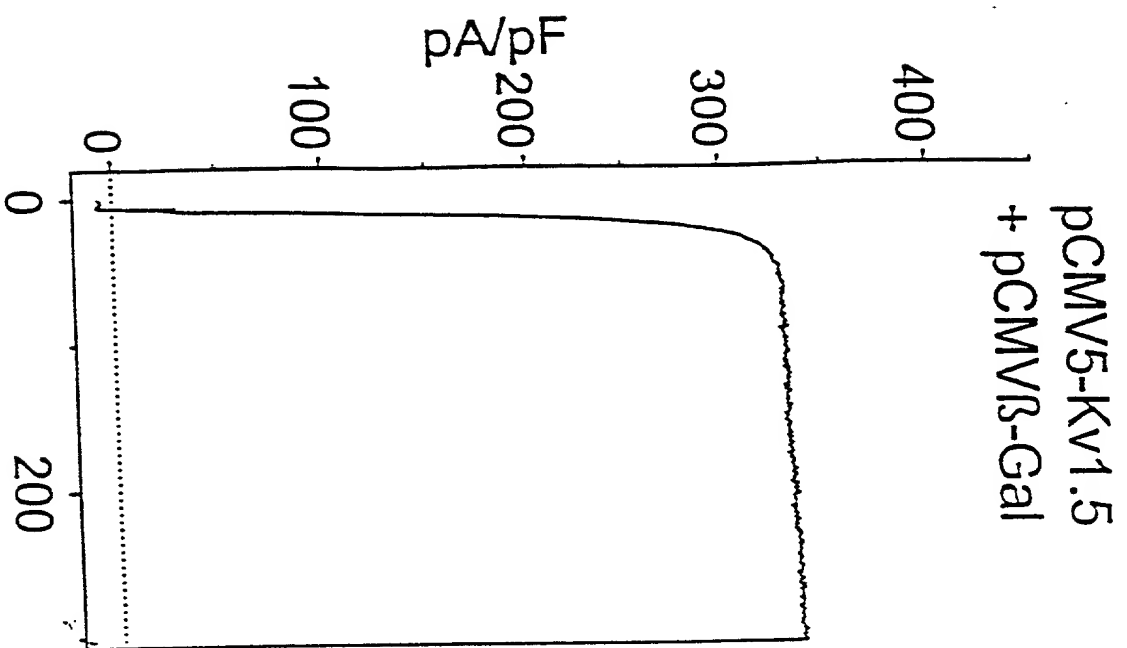


Fig. 9D

Time (msec)

Fig. 9E

Fig. 9F

19487659 4405443

Mean

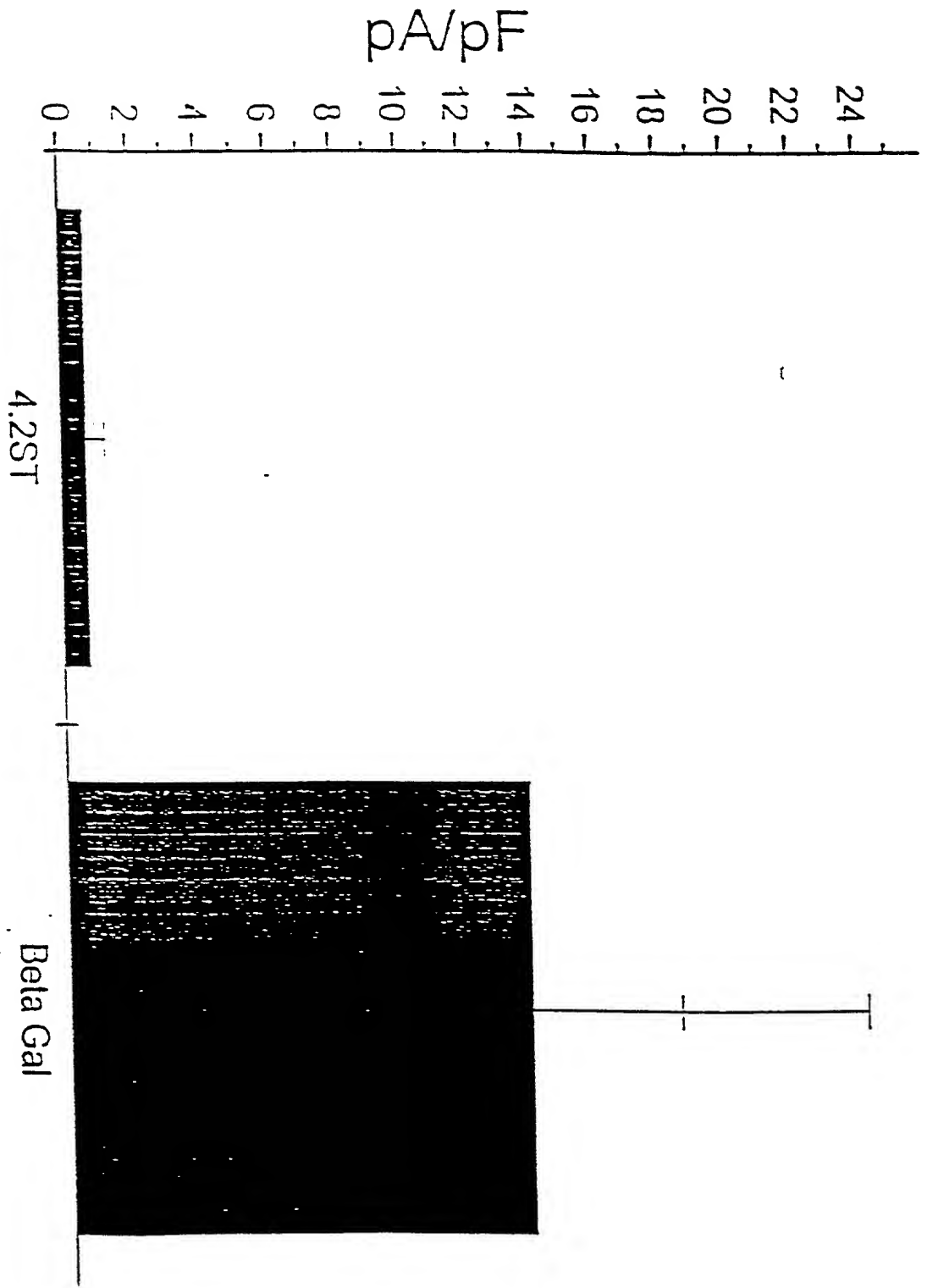


Fig. 10A

Fig. 10B

Fig. 11A

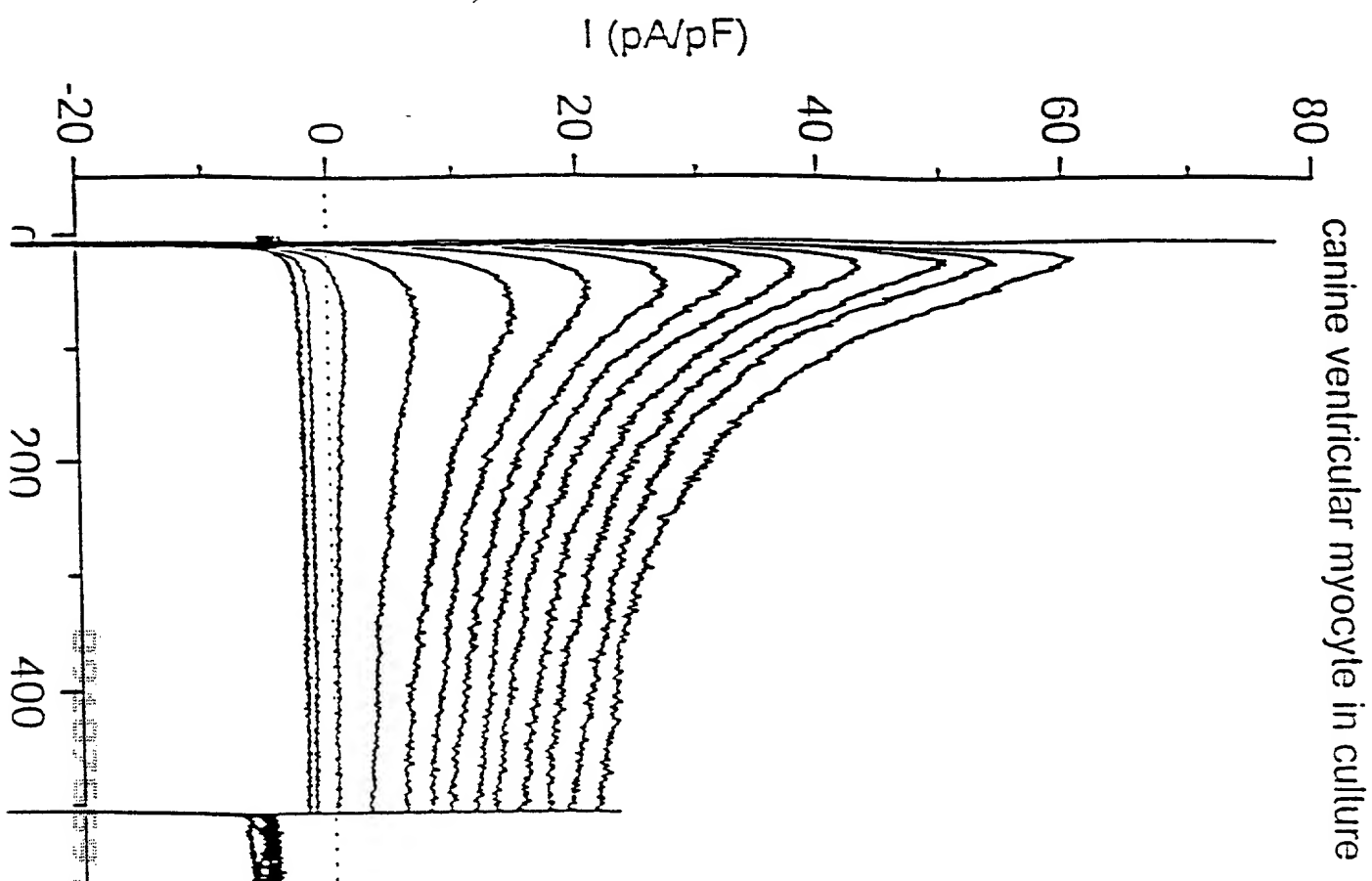
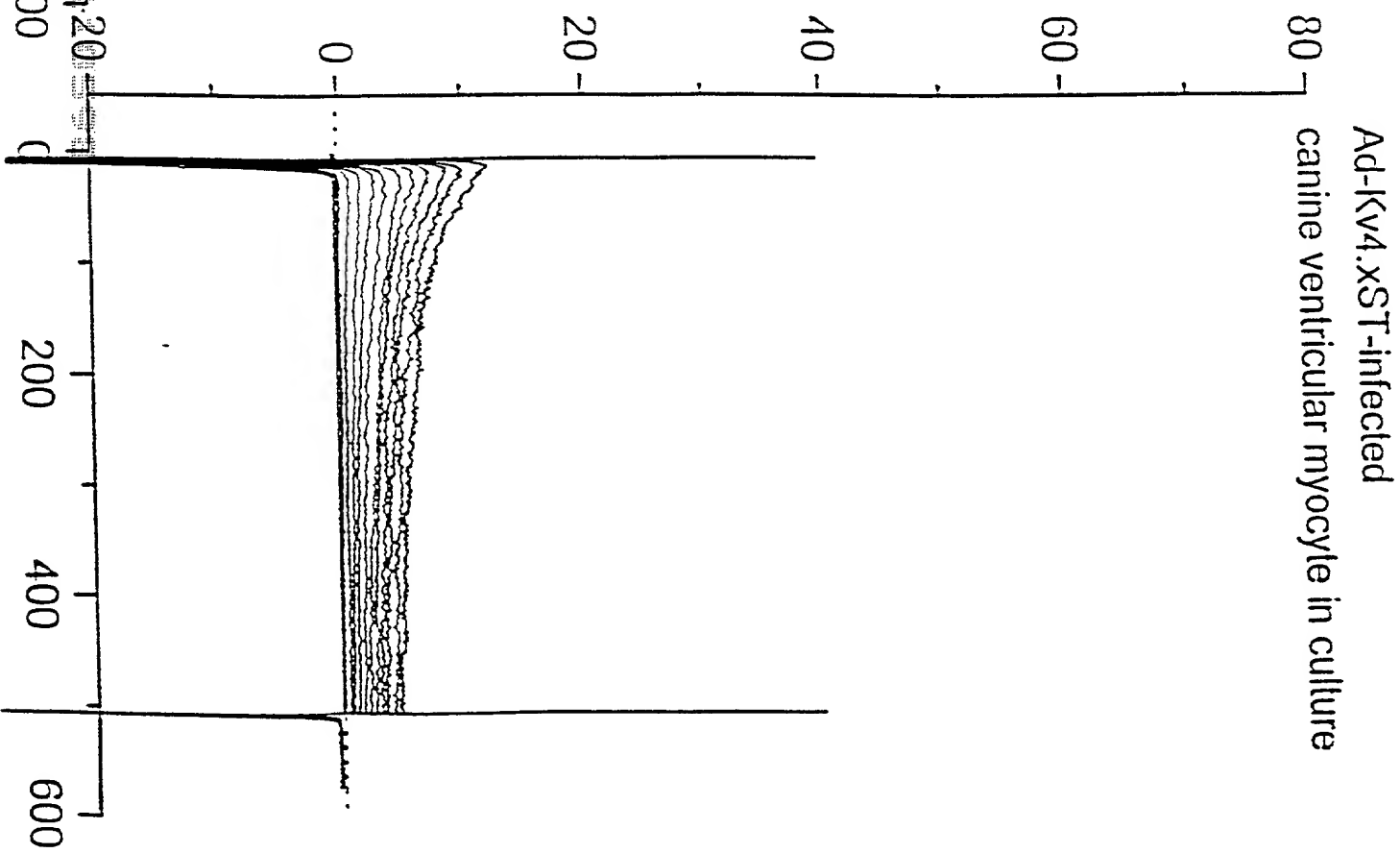


Fig. 11B



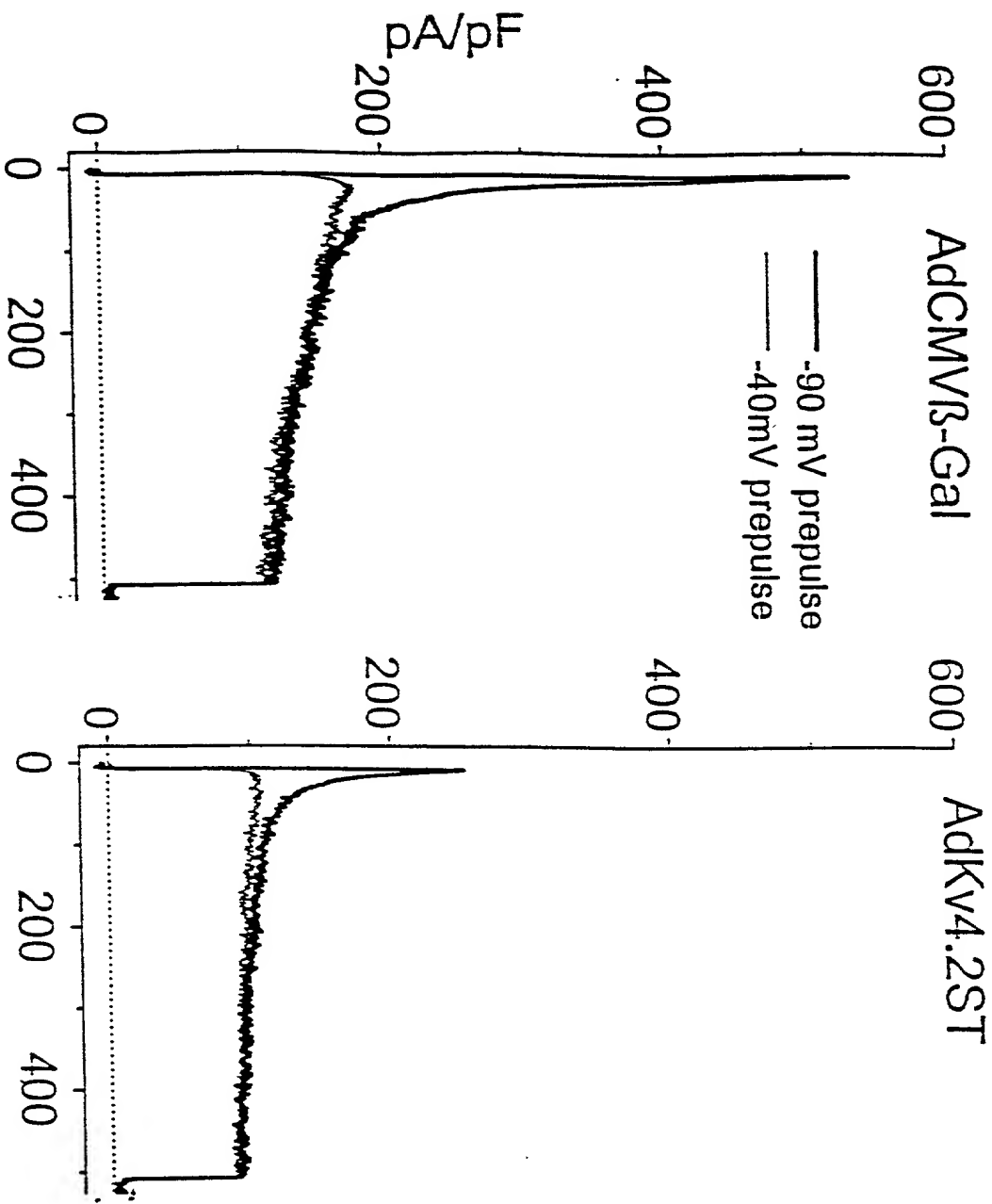
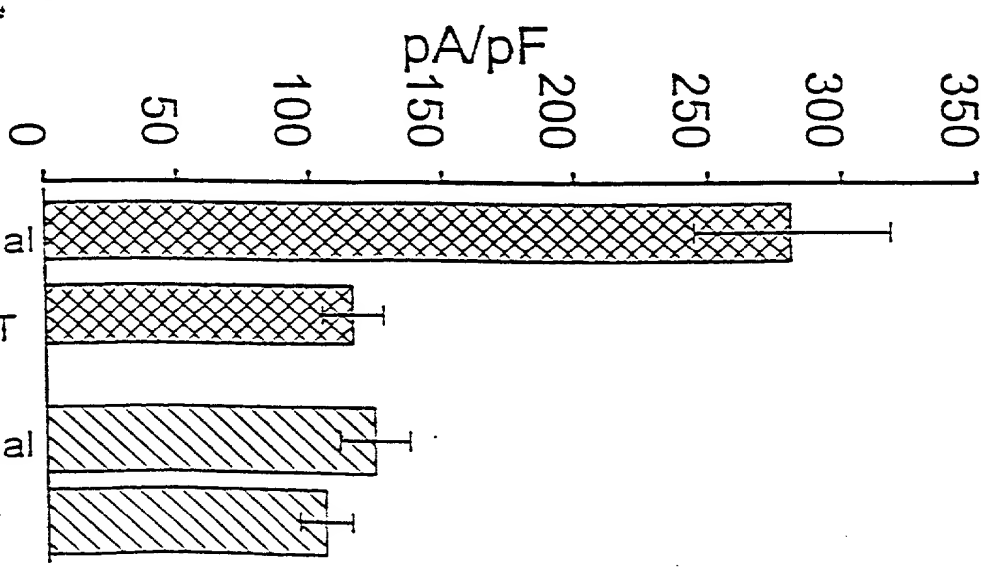


Fig. 12A

Time (msec)

Fig. 12B



Transient Component Maintained Component
Fig. 12C

09487669-440599

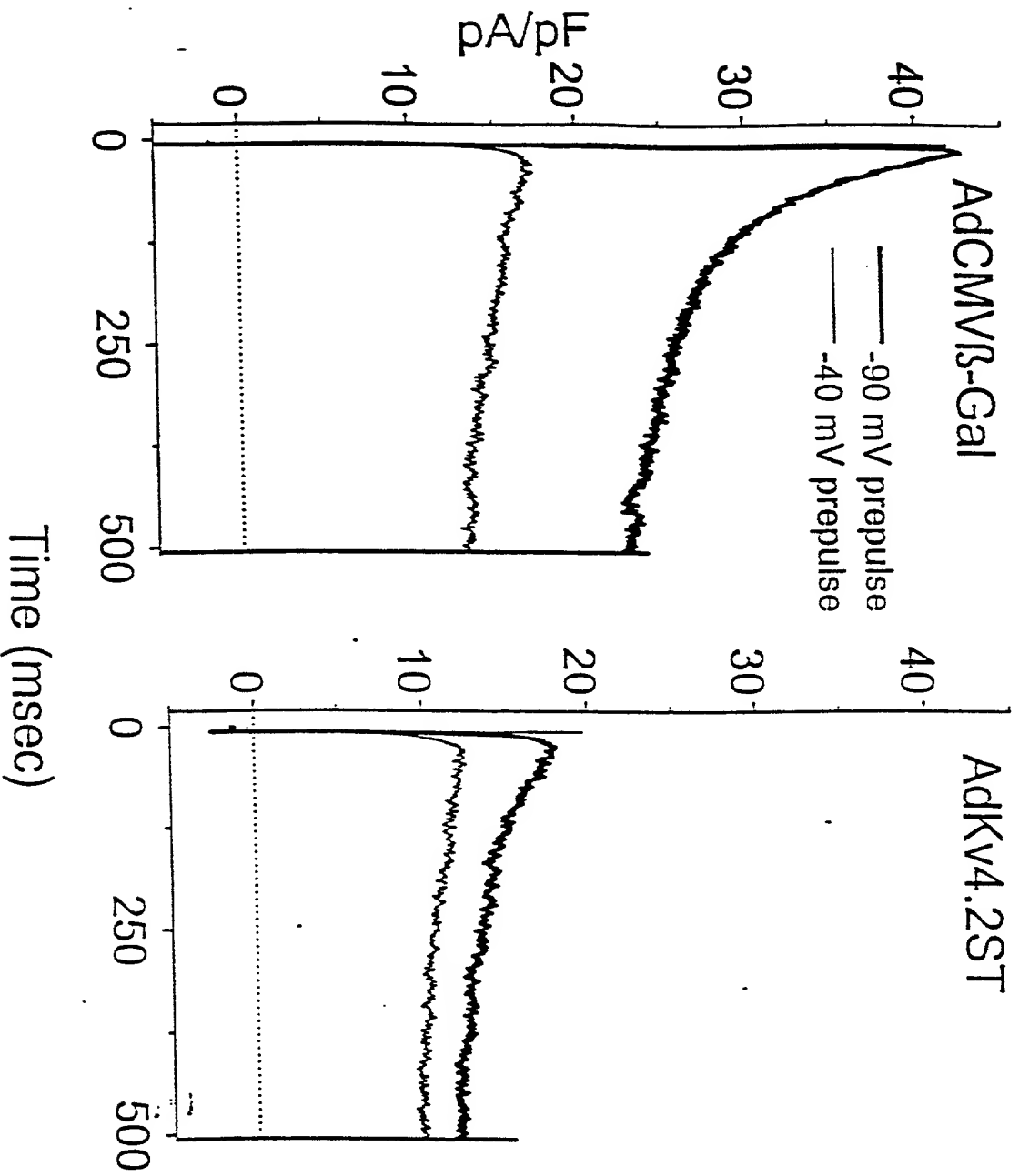


Fig. 13A

Fig. 13B

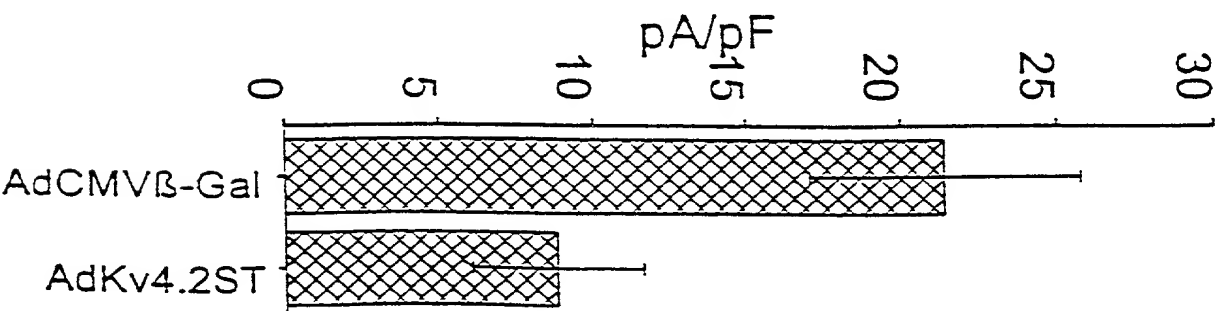


Fig. 13C

09187659-410599

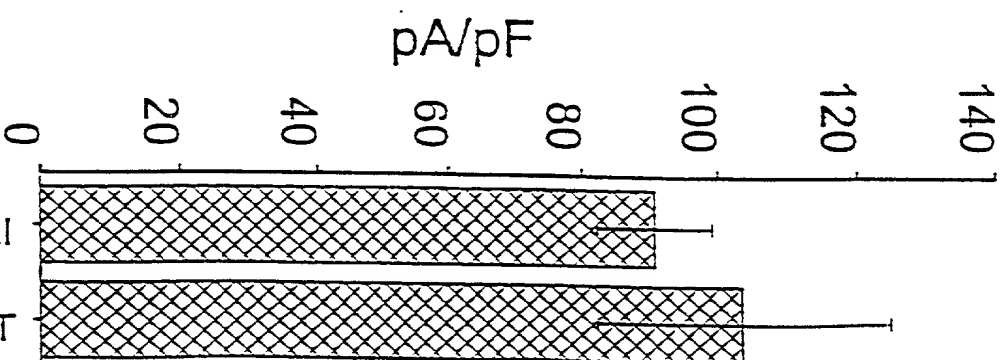
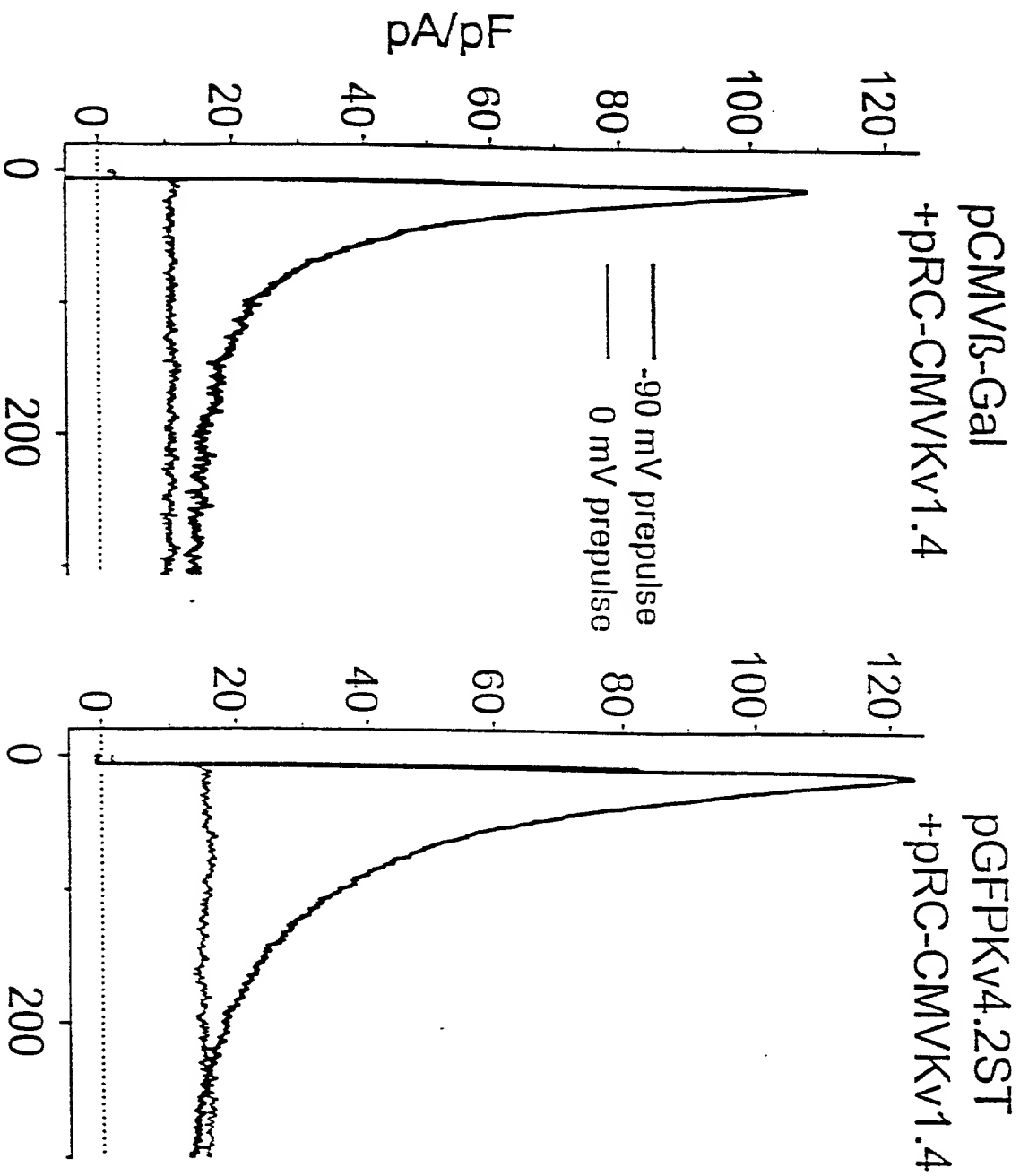


Fig. 14A

Fig. 14B

Fig. 14C

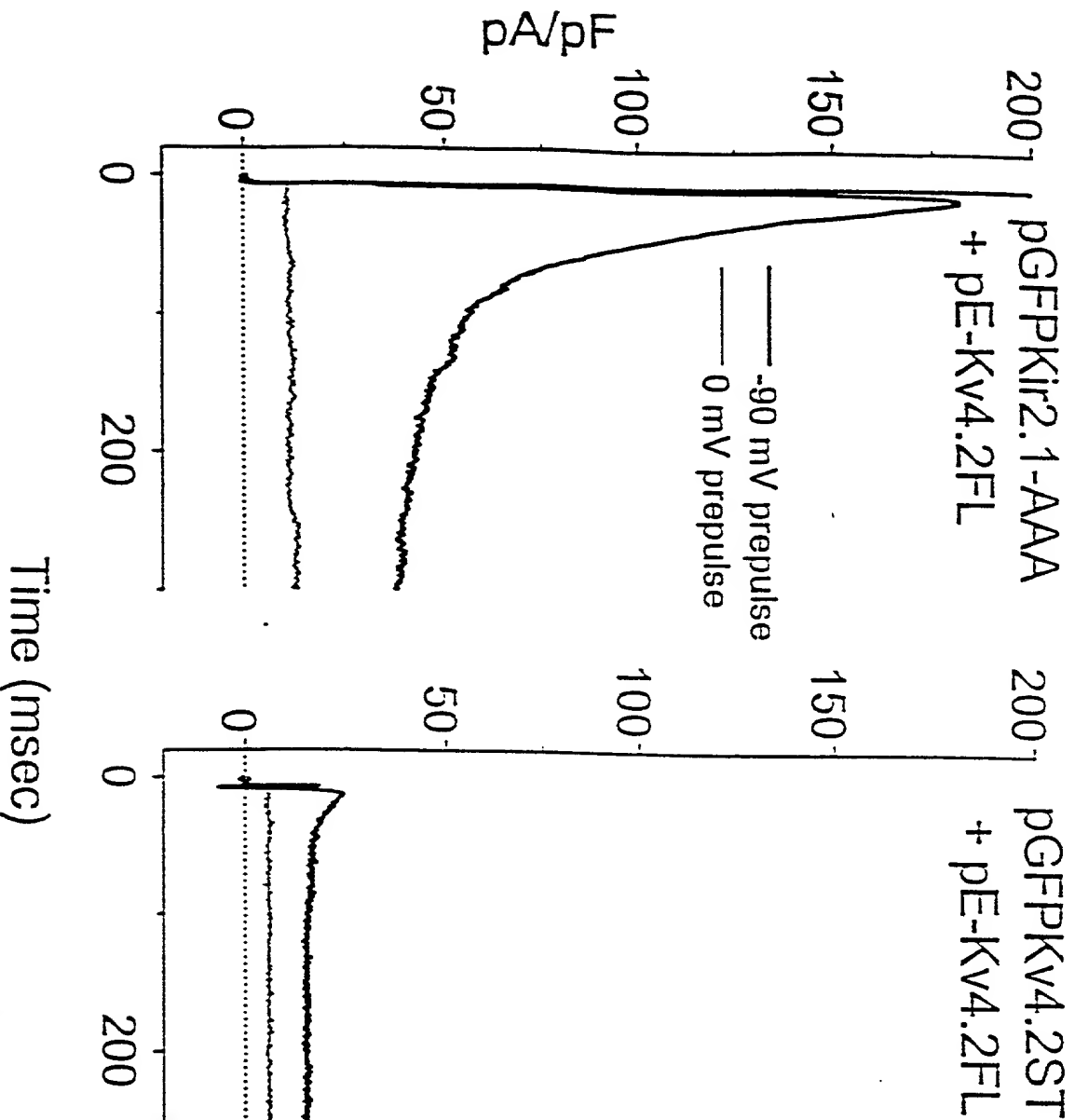


Fig. 14D

Fig. 14E

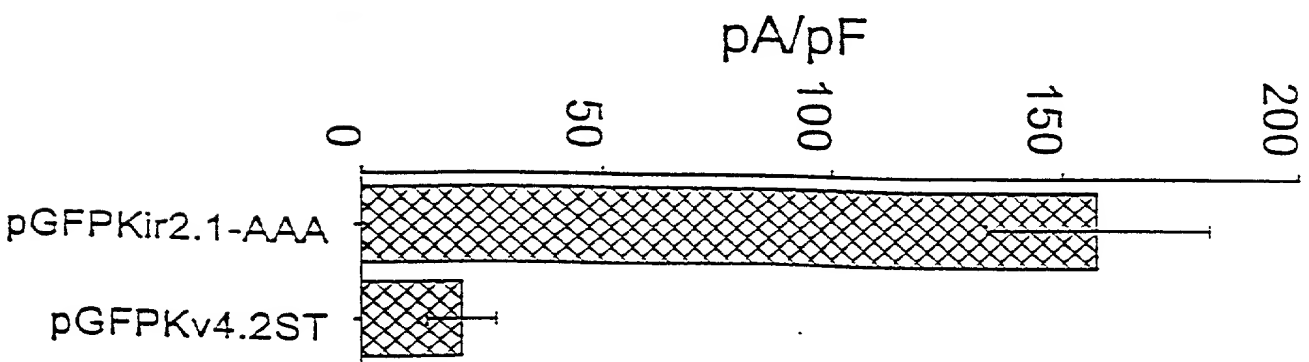


Fig. 14F

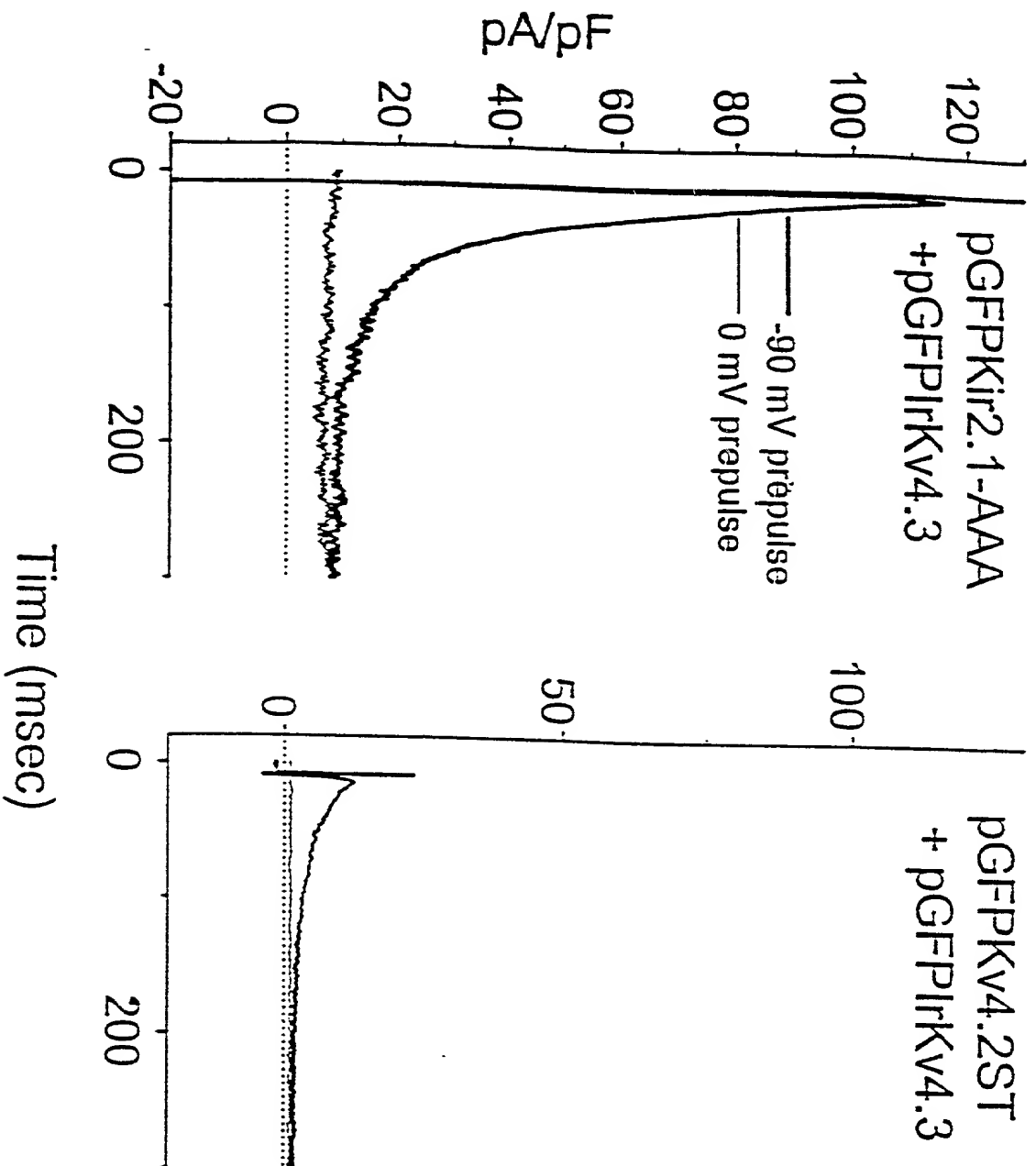


Fig. 14G

Fig. 14H

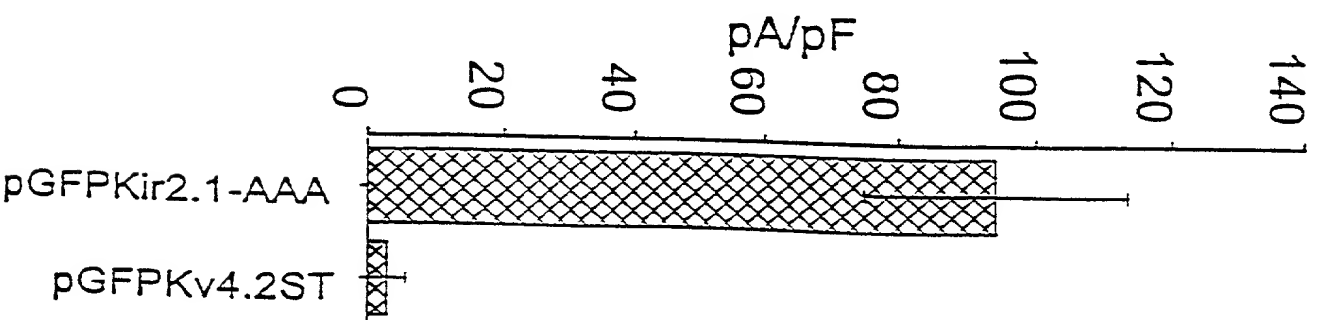


Fig. 14I

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Fig. 15A



Fig. 15B